Stabilization of solution formulation with protein-cosolvent interaction

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Contents

Chapter 1.

General Introduction

1.1. Pharmaceutical application of protein

Protein is an essential biological macromolecule in cellular systems. Many types of proteins play high-precision roles in many cellular functions such as metabolism [1], signal transduction [2], and facilitation of chemical reaction [3] which cannot be imitated by current industrial technology due to the functional origin. The function of a protein is based on its chemical structure and flexibility. A protein is a polypeptide comprising 20 naturally occurring amino acids that are required to fold into a unique three-dimensional structure for performing its function. Anfinsen's dogma [4] says that the unique structure of the protein is determined by its amino acid sequence. Each amino acid has the same backbone with different sidechains that have various properties, such as charge and hydrophobicity [5]. These simple differences make for versatile interactions and affect the complex energy landscape of protein folding [6]. Importantly, these interactions also enhance the structural flexibility of protein which is an essential factor for the function of the protein [7]. Altogether, these properties of protein make it difficult to mimic the functions of a protein with other artificial materials. Thus, industry regards a protein as an irreplaceable material for versatile purposes.

The function of proteins has been applied to many fields, such as material science [8], pharmaceutical science [9], food science [10], cosmetic science [11], and biology itself [12,13]. The pharmaceutical application of protein is a promising field because of its high selectivity and biocompatibility which are essential to avoid the side effects. To date, many proteins, such as antibodies, hormones, growth factors, and enzymes, have been commercialized for the treatment of various diseases that cannot be managed with conventional low-molecular-weight drugs [14]. Antibodies are one of the most promising proteins for pharmaceutical application [15]. Antibody function is based on antigen recognition at the antigen-binding fragment (Fab) and immune system activation by the fragment crystallizable (Fc) region [16]. The selectivity and affinity of the antibody can be modified by the substitution of amino acids in the variable Fab region [17], and the pharmacokinetic property of the antibody can also be modified in the Fc region [18]. Due to these

features, many types of monoclonal antibodies are now commercially available for the treatment of various target diseases, including cancer [19], asthma [20] and rheumatoid arthritis [21]. Accordingly, the market for antibody pharmaceuticals reached almost $75 billion in 2013 and continues to grow [22]. Recent developments in technology have enabled us to expand the function of the antibody. For example, a bispecific antibody can recognize two different antigens simultaneously [23], whereas an antibody–drug conjugate can selectively release a low-molecular-weight drug at a targeted site [24]. These advances in the pharmaceutical application of antibody will further benefit humanity.

1.2. Challenges for storage of pharmaceutical protein

Protein instability is the main obstacle for pharmaceutical application. The native structure of the protein is mainly stabilized by non-covalent bonds [25], with the exception of disulfide bonds [26]. Unlike covalent bonds, non-covalent bonds have only a marginal stabilizing effect on proteins. Although the marginal stability of proteins can induce the fluctuation for performing its function [7], protein is unstable in the presence of stress. In an aqueous solution, there are many types of stress, including pH shift, heat, shaking, and oxidation, which can unfold proteins and expose their hydrophobic region. The unfolding of protein causes a loss of its native function and the formation of its aggregate, which can cause disorders and unexpected immune reactions [27]. After it became commercially available, the aggregate of an erythropoietin peptide mimetic product was found to induce a fatal anaphylactic response in patients, forcing the manufacturer to withdraw it from the market [28]. Therefore, a reliable method to extend the duration of protein storage is needed.

Several approaches have been used to enhance the stability of proteins. Most proteins are more stable in an amorphous solid state than in a liquid state. Lyophilization, one of the most common methods used in the production of pharmaceuticals [29], successfully extends the shelf life of many pharmaceutical proteins with various excipients, including sugars [30,31], polyols [32], and synthetic polymers [33]. However, the preservation of proteins in an amorphous solid state necessitates the use of additional processes in drug manufacturing and delivery to patients. Before the lyophilized drug can be used, it must be reconstituted from the solid amorphous state back to the liquid state. This process is complicated and time-consuming and can cause aggregate formation [34,35]. Therefore, the manufacturer must investigate the optimal conditions for

aggregation suppression during the reconstitution process. The optimal conditions differ depending on the protein used, and protein pharmaceuticals with an extended shelf life become cost-prohibitive, in terms of both function and economics for the manufacturer and consumer. Although freezing protein solutions is a simpler preservation method than lyophilization because of the absence of the drying process, product quality still needs to be maintained under cold chain conditions, and the development of cold chain requirements for vaccines is very costly [36,37]. Therefore, freezing to preserve protein pharmaceuticals is also undesirable. The development of a method to overcome the weakness of pharmaceutical proteins in the liquid phase will profit not only our health but also our finances.

1.3. Strategy for stabilization of protein in solution

There are several types of methods to stabilize proteins in solution. The substitution of amino acids by recombinant technology is the most significant enhancement strategy for protein stability [38]. In this method, a specific site on the protein is modified with several intentions, such as reducing its hydrophobicity [39], deleting its aggregation-prone region [40], and creating a new interaction [41]. Another strategy is chemical conjugation with a synthetic polymer, such as polyethylene glycol (PEG) [42,43]. Although these methods can enhance the stability of the protein and increase the chance of it reaching commercialization, technical limitations exist, and another method is still required. The substitution of amino acids usually relies on existing empirical knowledge and requires much trial and error, making it difficult to find the optimal variant from a large number of mutant combinations. The chemical conjugation of a protein requires a complex production system for precise chemical reactions at target sites and for efficient purification, while the injection of PEGylated protein into humans induces the anti-PEG IgM response, which can accelerate protein clearance in the blood and impair the drug's efficacy [44]. These limitations may make the stabilization approach undesirable for pharmaceutical application. Therefore, another technology is needed to complement and enhance these approaches.

The stabilization of proteins can also focus on their exterior, i.e., the solvent. In solution, the surface residue of protein interacts not only with other residues but also with solvent molecules. There are many types of interactions between the protein's surface amino acid residue and the solvent, which affect protein stability

and function [45], and it is possible that altering these interactions can modulate the stability of the protein. Altering a solution with a cosolvent, also known as an additive, is a simple method to change the interaction at the protein surface. Mutagenesis or the conjugation of proteins can be combined with a cosolvent because of differing modification targets. The cosolvent effect on protein stability is often studied using a model protein, such as lysozyme [46–49] ribonuclease [50–52], β-lactoglobulin [53], and α-chymotrypsinogen [54]. The stabilization mechanism of the cosolvent is dependent on its affinity to the protein surface. Protein stabilizers, including sugar [55], polyol [56], and sodium sulfate [57], are preferentially excluded on the protein surface, causing energetic instability. In this circumstance, the protein favors its compact native structure to minimize the instability stemming from the protein surface. Solubilizing agents, including surfactants [58] and denaturants [59], favorably interact with the hydrophobic residues of proteins and inhibit protein association. Arginine shows a distinct mechanism of the inhibition of protein association via pi-cation and weak hydrophobic interaction [60]. The wise use of these types of additives and interaction will be helpful for pharmaceutical applications using proteins.

Although the base knowledge about the effect of cosolvents on protein stability is available, there are few studies on combining two or more cosolvents, and it should be investigated whether this mechanism can be adopted for stabilizing pharmaceutical proteins. Arginine suppresses the aggregation of lysozyme [46] but facilitates the association of phosphorylase kinase [61], and the effect of arginine can be enhanced using glutamate or aspartate [62–64]. It is possible that other combinations can also stabilize proteins. Therefore, understanding the question of cosolvents will help in the preservation of pharmaceutical proteins.

1.4. Objective of this thesis

The pharmaceutical application of protein is an important field of pharmaceutics. However, the intrinsic instability of proteins in solution is an obstacle. This thesis investigated the possibility of applying protein–cosolvent interaction research to the pharmaceutical field. Chapter 2 describes a method that can alter the effect of arginine using counterions. Chapter 3 discusses the effect of various cosolvents on the aggregation pathway of immunoglobulin. Chapter 4 shows that trimethylamine N-oxide (TMAO) can be used as a stabilizer for benzyl-alcohol-induced aggregation. Chapter 5 illustrates the behavior of two model proteins in an ethanol

solution to improve understanding of the disinfection mechanism of a non-enveloped virus. Subsequently, Chapters 6 and 7 present the overview and discussion of this thesis, including the future studies to be performed.

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Chapter 2.

Effect of Counter Ions of Arginine as an Additive for the Solubilization of Protein and Aromatic Compounds

2.1. Introduction

Arginine (Arg) is one of the versatile cosolvents (additives) in development of therapeutic or reagent proteins due to its ability to suppress protein aggregation without altering or destabilizing the tertiary structure of the protein [1]. It has been used for many applications including refolding enhancement [2–4], suppression of heat induced aggregation [5], reduction of the viscosity of concentrated protein solutions [6,7] and solubilization of aromatic compounds [8–12]. Molecular mechanisms underlying these effects have been proposed, e.g., the cation-π interaction between the guanidium group of Arg and aromatic groups of proteins or small organic solutes [10,11] and weak binding of Arg ions to the protein surface [13,14]. In these applications and analyses, Arg has been used at neutral or acidic pH and hence as a salt form, primarily chloride salt. Few studies were done with other salt forms [15,16].

Ions, especially anions, have specific effects on stability, solubility and aggregation of proteins in aqueous solution, known as Hofmeister series [17] or also as attraction pressure [18,19] and later developed into cavity theory [20]. Such ion-specific effects exist even on guanidium ion, as its denaturation effect differs between chloride and sulfate salts [21]. Molecular mechanisms governing ion-specific effects have been related to the strength of ionic hydration [22,23], different density of water molecules [24], and accumulation or exclusion of the ions from the protein surface [25]. It is thus highly likely that the effects of Arg can be modulated by anions. Recently, Izutsu et al. examined the effects of counter ions on the ability of Arg to stabilize proteins in frozen solutions and freeze-dried solid [26]. It was suggested that the interaction between multivalent counter ion and Arg plays an important role in protein stabilization [23]. Although the physical

state in consideration is different (solid state vs. liquid state), this argument is consistent with the observation by Trout et al. [27] that multivalent counter ion facilitates clustering of Arg, crowding out the protein-protein interaction and thereby suppressing aggregation. Here, I have initiated a systematic study on the effects of anions using small organic compounds. Previously, some researchers investigated the effects of ArgHCl on the solubility of aromatic compounds [8–11]. In this study, I examined the effects of various Arg salts on the solubility of such aromatic compounds as propyl gallate, phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) and reduce lysozyme and on refolding of the reduced lysozyme.

2.2. Material and methods

Materials

L-Arginine (Arg) was kindly provided by Ajinomoto Co., Inc. (Tokyo, Japan). Hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid, formic acid, citric acid (anhydrous), sodium hydroxide, sodium sulfate, sodium acetate, guanidium hydrochloride, guanidine sulfate, tyrosine, phenylalanine and tryptophan were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Sodium chloride was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Propyl gallate was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Hen egg white lysozyme and guanidium acetate were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). All chemicals used were of reagent grade and used as received. All arginine salt forms were prepared by titrating an aqueous solution containing Arg base with the above acids. Although addition of water to Arg base resulted in suspension, acid titration of the suspension lead to clear solution due to protonation of basic groups of Arg. It should be noted that ArgH(H_2PO_4) slowly phase-separated due to low solubility of monovalent phosphate

Solubility measurement of aromatic compounds

The solubility of aromatic compounds, i.e., propyl gallate and aromatic amino acids, in the absence and presence of additives at pH 4.8 was measured as described in the previous studies [9,10]. Higher pHs were also tested with difficulty in maintaining a constant pH: dissolution of propyl galate resulted in significant pH reduction at such higher pH values. Propyl gallate and aromatic amino acids were transferred into test tubes,

to which 0.5 ml of test solvents were added. The suspension was heated at 50°C for 1 hour with frequent vortexing to completely dissolve the solute compounds. The solutions were incubated at 25°C for 3 days with frequent vortexing, leading to development of suspension. Subsequently, the suspension was centrifuged at 25°C and 18,800 g for 30 min to obtain a supernatant saturated with the solutes. After appropriate dilution of the supernatant with 10 mM citrate buffer (pH 4.8), the absorbance of the supernatant was determined at 273 nm, 275 nm, 257 nm and 279 nm for propyl gallate, tyrosine, phenylalanine, tryptophan, respectively. The absorbance spectrum was recorded using an ultraviolet-visible (UV-VIS) spectrophotometer (ND-1000; NanoDrop Technologies Inc., Wilmington, DE, USA) and converted to the concentration on the basis of the standard curve determined for each solute compound. Solubility was determined in triplicate from which the averages and standard errors were obtained.

Estimation of transfer free energy

The transfer free energy ΔG_{tr} of the propyl gallate from sodium salt solution to arginine salt solution at a given additive concentration, e.g. from 1 M sodium chloride solution to 1 M Arg chloride solution, was calculated according to the following equations:

$$\Delta G_{tr} = \mu_a^0 - \mu_s^0 = -RT \ln(x_a / x_s) \cdots (1)$$

$$\begin{cases} \mu_a = \mu_a^0 + RT \ln(x_a) \\ \mu_s = \mu_s^0 + RT \ln(x_s) \end{cases} \cdots (2)$$

$$\begin{cases} x_a = n_{g,a} / (n_{g,a} + n_{H_2O,a} + n_{a,a} + n_{c,a}) \\ x_s = n_{g,s} / (n_{g,s} + n_{H_2O,s} + n_{a,s} + n_{c,s}) \end{cases} \cdots (3)$$

In the equation (1) to (3), μ_a and μ_s are the chemical potentials of the alkyl gallate in the presence of Arg salt (a) and sodium salt (s), respectively, while μ_a^0 and μ_s^0 are the corresponding standard chemical potentials. The transfer free energy of the propyl gallate from the sodium salt solution to the arginine salt solution can be calculated from the solubility of the propyl gallate in the respective solutions x_a and x_s, expressed as the mole

fraction solubility of the propyl gallate in the presence of the additives. The mole fraction concentration is calculated using $n_{i,s}$ and $n_{i,a}$, which correspond to the molarity of the component i at saturation in the presence of the additives. Subscripts g, H_2O, a, c are used to express the propyl gallate, water, additive and counter ion, respectively. The activity coefficient was considered to be close to unity because of poor solubility of the alkyl gallate. *R* and *T* correspond to the universal gas constant and absolute temperature, respectively.

Solubility measurement of unfolded lysozyme.

Reduced carboxymethylated lysozyme (RCM-Lyz) was prepared as previous study [28]. Briefly, lysozyme was solubilized at 20 mg/ml in 100 mM Tris-HCl buffer, 8 M guanidine-HCl, 40 mM DTT, 1 mM EDTA and incubated at 37°C for 3 hours. Then, 100 mM iodoacetic acid was added to the solution and adjusted the pH to 8.0 by 5 M NaOH. The mixture was incubated at room temperature for 3h in the dark and subsequently dialyzed against 10 mM HCl for 1 day. After dialysis, the sample solution was freeze-dried. The resultant RCM-Lyz powder was suspended into the test solutions containing 1M Arg salt (pH 9.5) at room temperature for 3 days. After incubation the solution was centrifuged at 18,800 g for 30 min and the absorbance of the supernatant was measured. The solubility was calculated from the absorbance at 280 nm by ε= 2.37 for denatured lysozyme [29].

Refolding assay

Refolding experiments were carried out as previously described [30] with slight modifications. Lysozyme was reduced and denatured at 40 mg/ml in a solution containing 6 M Gdn, 1 mM EDTA, 40 mM DTT and 100 mM Tris-HCl buffer (pH 8.0) and incubated for 2 hours at 37°C. The denatured and reduced lysozyme was diluted 40-fold with an appropriate refolding buffer containing Arg salt and GSH and GSSH. The final concentrations of each ingredient in the refolding mixture are 1 mg/ml lysozyme, 150 mM Gdn-HCl, 1 mM DTT, 1 M Arg salt, 5 mM GSH, 5 mM GSSG, 1mM EDTA and 100 mM Tris (pH 8.0). The diluted solutions were vortexed for 2 s and incubated at 25°C for 15 h without shaking. After incubation, the sample solutions were centrifuged at 18,800 g for 30 min to remove the aggregates. After centrifugation, 10 μl of supernatant was mixed with 1490 μl of 0.3 mg/ml *Micrococcus Luteus* solution containing 50 mM phosphate buffer (pH 7.0) and monitored

the absorbance at 600 nm using a V-630 UV-vis spectrophotometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan). The refolding yield was determined from the initial velocity of decreasing absorbance, which was compared with 1 mg/ml native lysozyme.

2.3. Results

Solubility of propyl gallate in different Arg salt solution as a function of additive concentration.

The solubility of propyl gallate used as a model compound was determined as described in the method section. Figure 2.1 shows the solubility of propyl gallate in the presence of various Arg salts, i.e., acetate, formate, sulfate, chloride, citrate and phosphate, as a function of additive concentration at pH 4.8. All arginine salts increased the solubility of propyl gallate concentration dependently. The magnitude of solubilization effect depended on anionic species. The effect of ArgHCl, which is commonly used, was moderate, increasing the solubility from 3.5 to 8.8 mg/ml at 1 M. ArgH(acetate) was most effective with the solubility reaching 19.9 mg/ml at 1 M followed by ArgH(formate) and ArgH(citrate), resulting in 13.4 and 10.6 mg/ml at 1 M additives. The latter two Arg salts were still more effective than ArgHCl. On the contrary, $Arg(sulfate)_{1/2}$ and $ArgH(H_2PO_4)$ were less effective than ArgHCl, resulting in 6.8 mg/ml and 7.5 mg/ml at 1 M. These results indicate that the type of anionic species greatly influence the solubilization effects of arginine. The order of ArgHCl > $ArgH(sulfate)_{1/2}$ = $ArgH(H_2PO_4)$ in increasing the solubility appears to be consistent with the Hofmeister series. Thus, stronger hydration otential of the latter two anions or their stronger Arg clustering proposed by Trout et al. [24] can be used to explain different solubilization effects of these three Arg salts. The stronger solubilization effects of ArgH(acetate), ArgH(formate) and ArgH(citrate) cannot be readily explained from both mechanisms and may be related to the fact that they have caroboxyl groups.

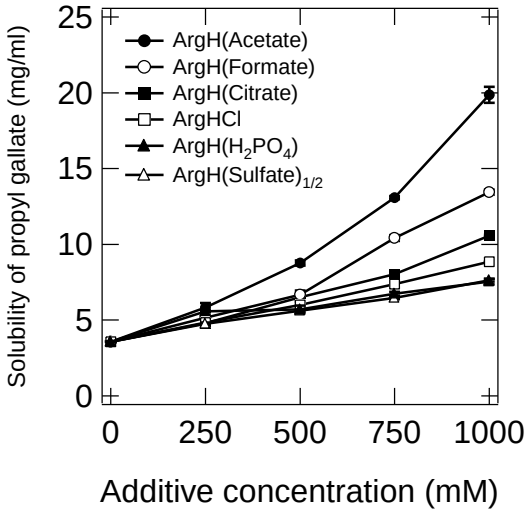

Figure 2.1. Solubility of propyl gallate in the absence and presence of arginine salt as a function of additive concentration. All solutions contained 10 mM citrate buffer (pH 4.8). The measurements were performed three times, and the error bars indicate the standard deviation of the mean.

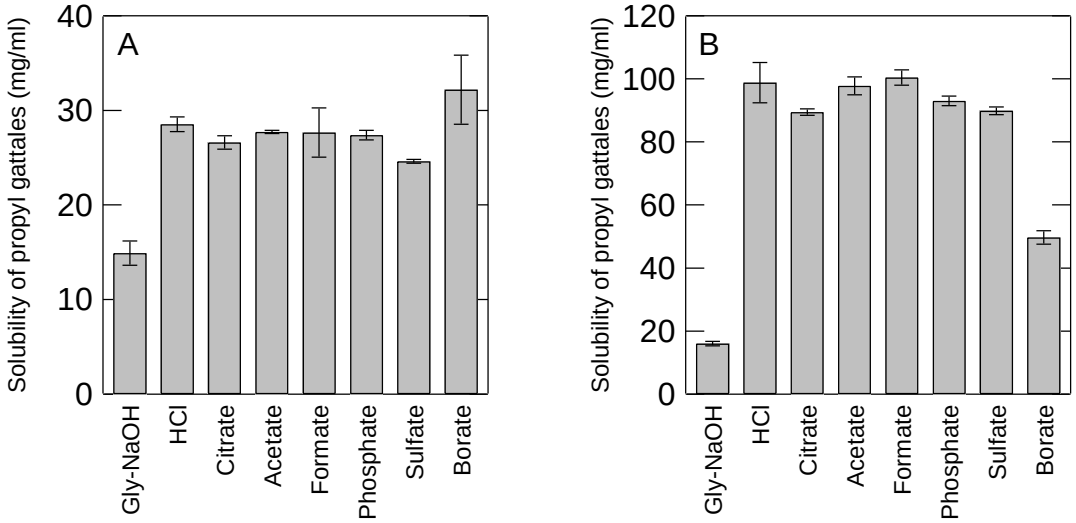

Figure 2.2. Solubility of propyl gallates in the presence of 100 mM(A) or 500 mM(B) arginine salt at pH 9.6 except for Gly-NaOH buffer. These solutions were prepared from 1 M stock solution by desalination water (DW). The measurements were performed three times and the error bars depict the standard deviation of the mean.

I have also attempted the same experiment at pH 9.6, at which Arg itself can serve as a buffer. However, I was unable to determine the propyl gallate solubility at a constant pH of 9.6 is high due to ionization of phenol group of propyl gallate and thereby pH changes (Figure 2.2). It should be noted that a similar experiment can be done at or near pH 9.6 using solutes that have no dissociable groups around this pH.

Comparison the solubilization effect of other salts with Arg salt

Whether the observed effects of anionic species on propyl gallate solubility is specific to Arg salt or universal was tested using sodium salt and guanidium salt. I chose the acetic, hydrochloric and sulfuric ion as weak, monovalent and multivalent anion, respectively. Figure 2.3A shows the solubility of propyl gallate in the presence of each sodium salt. Sodium chloride and sodium sulfate decreased the solubility of propyl gallate concentration dependently with the sulfate salt slightly more strongly. On the contrary, sodium acetate increased the solubility of propyl gallate. The observed order, acetate > chloride > sulfate, is identical to the order for their Arg counterpart, suggesting an identical mechanism operating on their effects on propyl gallate solubility. Regardless of cation species (sodium vs. Arg), their anionic species affects the propyl gallate solubility similarly. Figure 2.3B shows the effects of guanidine salt on the solubility of propyl gallate. All guanidine salts increased the solubility of propyl gallate. The magnitude of solubilization effect of guanidine salts increased in the order of acetate > chloride > sulfate, again consistent with the order observed for Arg and sodium salts.

I next compared the effects of Arg and sodium salts of the same anionic species by dividing the propyl gallate solubility in Arg salt solution by the corresponding value in sodium salt solution. Namely, it measures the rate of solubility increase by replacing sodium with Arg as a cation. Figure 2.4 shows the results of such a calculation. Regardless of anionic species, the ratio is greater than 1, indicating that the solubility of propyl gallate is universally greater with Arg than sodium, consistent with the established ability of Arg to increase the solubility of proteins and small organic compounds. The solubility ratios nearly fall on the same curve for these three anions, meaning that the enhanced solubility by Arg over sodium is independent of the anionic species. It thus appears that the solubilization effects of Arg ion is independent of the anionic species.

Transfer free energy of propyl gallate from sodium salt solution to Arg salt solution was calculated from the solubility ratio as described in the Method section. The transfer free energy, shown in figure 2.5, was negative for all cases. For example, the free energy of the propyl gallate decreased ~3 kJ/mol by transferring 1 mol/L of propyl gallate from 1 M sodium salt solution to 1 M arginine salt solution independent of the anionic counter ions. At any concentration, the transfer free energy is negative, meaning that the interaction of propyl gallate is more favorable with Arg than sodium ion and is not affected by the anionic species.

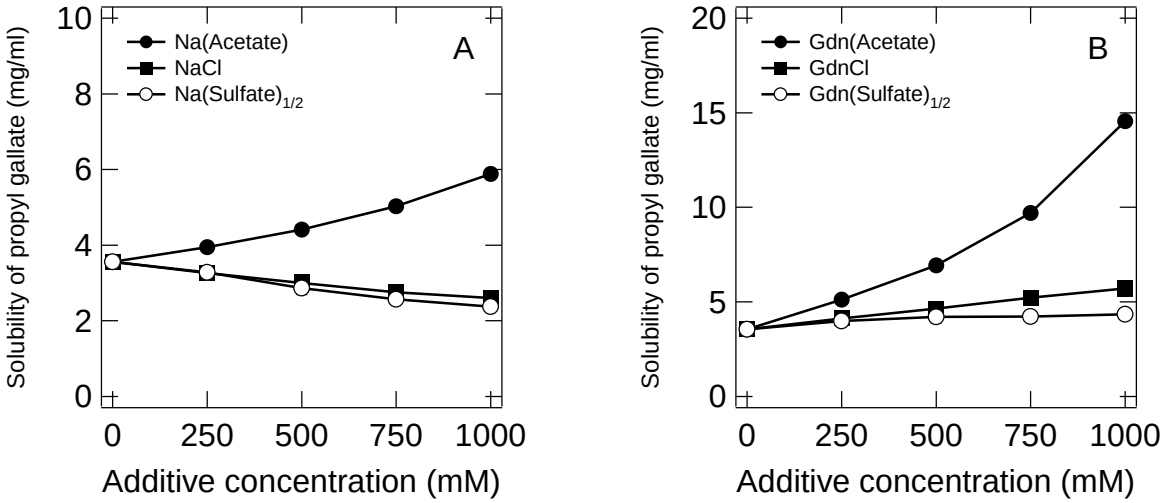

Figure 2.3. Solubility of propyl gallate in the absence and presence of (A) sodium salt and (B) guanidium salt as a function of additive concentration. All solutions contained 10 mM citrate buffer (pH 4.8).

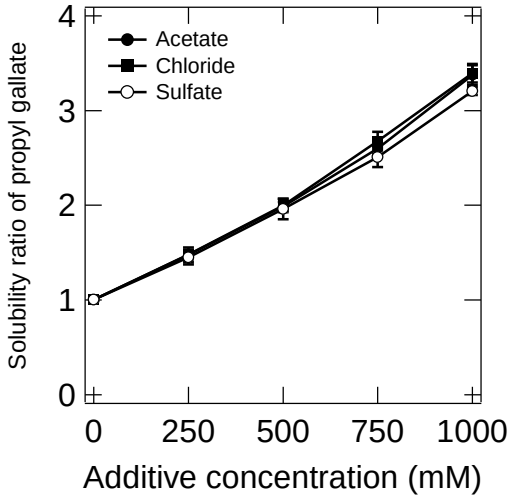

Figure 2.4. Ratio of the solubility of propyl gallate in Arg salt solution to that in sodium salt solution at a given additive concentration.

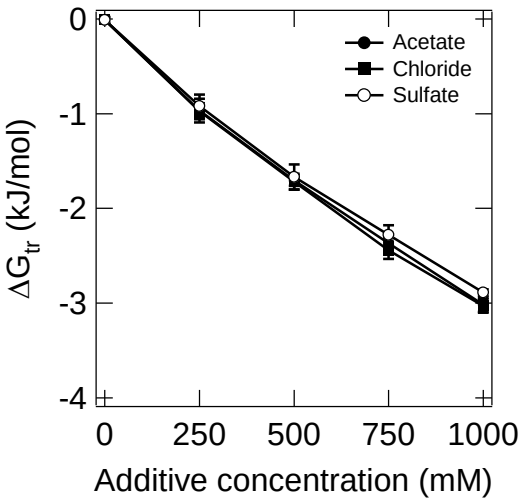

Figure 2.5. Transfer free energy of propyl gallate from sodium salt solution to Arg salt solution at a given additive concentration.

The effect of counter ion on the solubility aromatic amino acid

It was suggested above that there may be specific interactions between acetate and propyl gallate, as this anion was highly effective in increasing the propyl gallate regardless of the cationic species, i.e., whether Arg, Gdn or sodium. Thus, whether acetate effect is universal or specific to propyl gallate was tested with aromatic amino acids, namely Tyr, Phe and Trp, using Arg salts. Figure 2.6 shows the solubility change of these amino acids when transferred from buffer solution to Arg salt solutions at 1 M. It is evident from figure 2.6 that Arg(acetate) is more or less comparable with ArgHCl, demonstrating a unique nature of propyl gallate with regard to the interaction with acetate anion. In fact, with Tyr and Phe, the solubilization effects of 1 M ArgHCl, $Arg(sulfate)_{1/2}$ and Arg(acetate) were comparable. With Trp, the order was ArgHCl > $Arg(sulfate)_{1/2}$ > Arg(acetate), qualitatively different from the order observed for propyl gallate. Interestingly, all these Arg salts were marginally effective on the Phe solubility, suggesting that possible favorable interaction of Arg is weak against Phe.

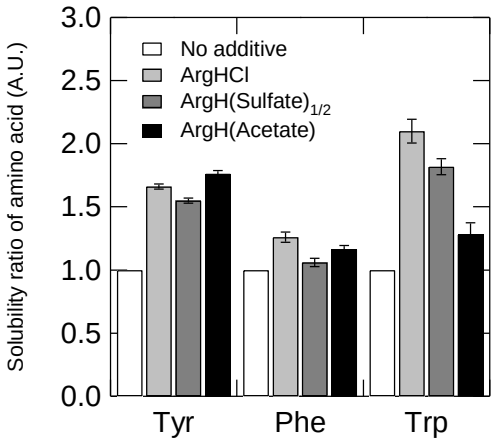

Figure 2.6. Ratio of the solubility of Tyr, Phe and Trp in 1 M Arg salt solution to that in its absence. All solutions contained 10 mM citrate buffer (pH 4.8).

Effect of Arg salt on protein solubilization and refolding

As mentioned above, the solubility of small aromatic compounds were influenced not only arginine but also their counter ions. It may be possible that the solubility of proteins is also similarly affected by Arg salts. Therefore, I next examined the solubility of RCM-lyz in Arg salt solutions. ArgHCl, as shown in figure 2.7, increased the solubility of RCM-lyz from 0.03 mg/ml to 1.54 mg/ml at 1 M. Other Arg salts, Arg(sulfate)$_{1/2}$ and Arg(acetate), were also effective, increasing the solubility of RCM-Lys to 0.45 and 0.4 mg/ml, but which were far less than the solubility in ArgHCl. It is thus evident that for protein solubility, ArgHCl is more effective than Arg(sulfate)$_{1/2}$ and Arg(acetate), an observation different from the propyl gallate solubility.

Figure 2.8 shows the refolding yield of lysozyme at pH 8.0. In the absence of additives, the refolding yield was only 6%. ArgHCl increased the refolding yield to 65% at 1 M. Consistent with the solubility of RCM-Lyz, sulfate ion and acetate ion greatly suppressed the refolding effectiveness of ArgHCl, resulting in 7 and 18% refolding yield.

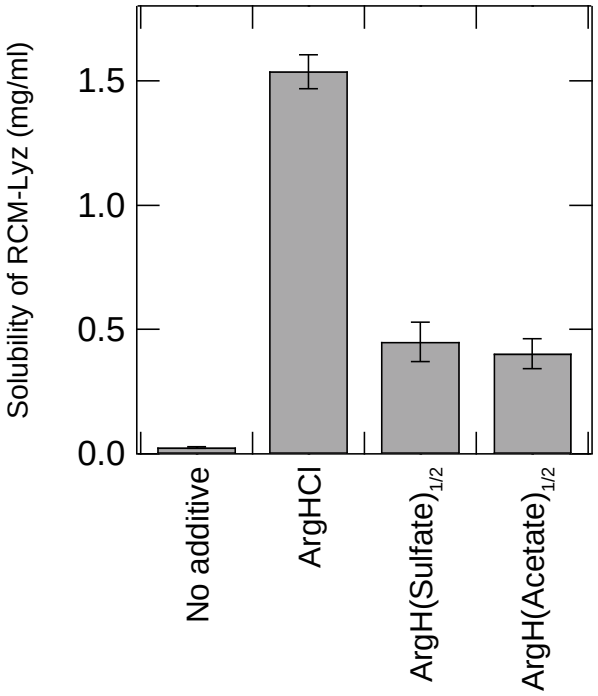

Figure 2.7. Solubility of RCM-lyz in the absence and presence of 1 M Arg salt. All solutions contained 10 mM Tris buffer (pH 9.5).

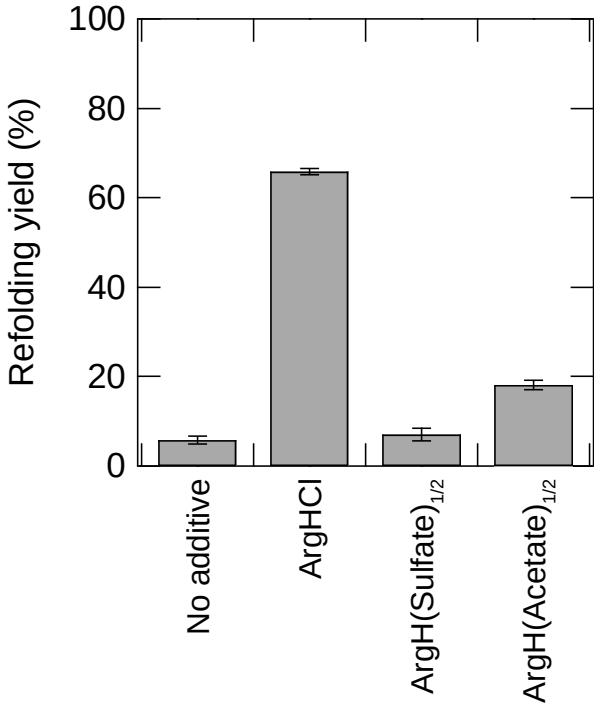

Figure 2.8. Refolding yield of lysozyme in the absence and presence of 1 M Arg salt. All solutions contained 150 mM Gdn-HCl, 1 mM DTT, 5 mM GSH, 5 mM GSSG, 1mM EDTA and 100 mM Tris (pH 8.0).

2.4. Discussion

This study examined how counter anions modulate the solubilization effects of arginine and also how the counter cations alter the effects of anionic species in the Arg, sodium and guanidine salts. Arg salts increased the solubility of the propyl gallate in the order of acetate > formate > citrate > chloride > phosphate > sulfate, of which the first three acids do not follow the Hofmeister series (Figure 2.1). Among these three acids, acetate was particularly effective in increasing the propyl gallate solubility with not only Arg but also sodium and guanidine, suggesting unique interaction between propyl gallate and acetate. This was supported by the observation that Arg(acetate) was not special compared with sulfate and chloride against the solubility of Tyr, Phe and Trp. Since all these solutes possess aromatic ring structure, the unique interaction between acetate and propyl gallate may be due to propyl group in propyl gallate. However, acetic acid, formic acid and citric acid have all carboxyl groups, of which the latter two were significantly less effective. Previously, molecular dynamics simulation suggested favorable hydrogen bonding interactions between hydroxyl group of phenol and carboxylate anion [31]. If this is an only mechanism of acetate on propyl gallate solubility, then Arg(acetate) should also increase the Tyr solubility, inconsistent with only small increment of Tyr solubility by acetate over chloride. Thus, there must be additional factor responsible for the acetate effects on propyl gallate solubility. Regardless of the mechanism, Arg(acetate) greatly increases the solubility of propyl gallate, which is used as an antioxidant, and hence may have a commercial value according to the previously proposed arginine assisted solubility system (AASS) [10].

With regard to ArgHCl and $Arg(sulfate)_{1/2}$, it appears that the solubility of propyl gallate decreased in that order. The propyl gallate solubility also decreased with sodium or guanidine chloride and sodium or guanidine sulfate, more so for sulfate, meaning that their effects are independent of the cationic species. Such anion-specific effects on protein solubility were first observed by Hofmeister and have been explained by surface tension increment caused by ions [18–20] or ion hydration [24]. When a solute molecule is introduced into an aqueous solution, solvent water generates a cavity to accommodate the solute and creates an interface between bulk water and the solute. If the surface tension of aqueous solution is low, less energy is required to make a cavity, resulting in higher solubility. If the surface tension is high, greater energy is required.

The solubility of RCM-Lyz increased in Arg, whose effects are modulated by anionic species (Figure 2.7). This effect can be explained by the interaction of Arg with aromatic amino acids in lysozyme (Figure 2.6). Hen egg white lysozyme has three tyrosines, three phenylalanines and six tryptophans [32]. Favorable interactions between Arg and these aromatic side chains should play a role in increased solubility of RCM-Lys. Such Arg effects are most favorable with chloride ion, compared with sulfate and acetate ions.

The dependence of refolding yield of lysozyme on counter ion is generally the same as the dependence of RCM-Lyz solubility, i.e., ArgHCl is most effective in increasing the refolding yield (Figure 2.8). Previously, it is reported that sodium sulfate stabilizes the intermediate state of protein [33]. On the basis of this, I consider that sulfate decreases the refolding yield of lysozyme by stabilization of the aggregates and the intermediate states. Although sulfate also stabilizes the native state by salting-out effect, higher energy barrier between the native state and the intermediate state makes kinetics of refolding slower and facilitates aggregation. Compared with sulfate, acetate has a weaker salting-out effect [25], which may be consistent with a slightly higher refolding yield by Arg(acetate) than Arg(sulfate). and Arg can exhibit small effect. Interestingly, Trout reported that Arg(sulfate)$_{1/2}$ more effectively suppresses thermal aggregation of α-chymotrypsinogen than Arg(acetate) and ArgHCl [27]. This discrepancy is perhaps due to the different states of the proteins used. Thermal aggregation may be more effectively suppressed by stabilizing the native state, the effect conferred by sulfate salt. Refolding, on the contrary, may solution need not only aggregation suppression but also a proper folding pathway, which can be afforded by ArgHCl.

In summary, I showed that the selection of counter ions can modulate the effects of Arg on the solubility of aromatic compounds and reduced protein as well as on refolding of the reduced lysozyme. Such modulation is independent of the interaction between Arg and aromatic compounds. Namely, the anionic species modulate the propyl gallate solubility independently. ArgHCl has been commonly used in many biological and biotechnological applications [34,35]. Here, I have shown that the effects of Arg may be further enhanced using different anionic counter ions depending on the target solute.

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Chapter 3.

Thermal Aggregation of Human Immunoglobulin G in Arginine Solutions: Contrasting Effects of Stabilizers and Destabilizers

3.1. Introduction

Antibody is one of the most important classes of biopharmaceutics owing to its high specificity and biocompatibility [1,2]. Antibodies are composed of multiple globular domains that assemble into a Y-shaped structure, generating multiple binding sites to themselves and other molecules [3]. Their Fab region is responsible for antigen-specific binding, and the Fc region is responsible for receptor binding [3]. Although this complex structure results in antigen specificity of the antibody with few side effects, it may be a potential source for aggregation [4–6]. Despite its high solubility, the antibody tends to form aggregates both reversibly and irreversibly due to various stresses during manufacturing, shipping, and storage processes [7]. The aggregation of proteins is known to cause unexpected immunogenic reactions [8]. Therefore, understanding the mechanism of antibody aggregation and the method to prevent aggregation is critical for developing safer products.

Protein aggregation can occur in two different manners, i.e., via aggregation of native or nonnative structures [9,10]. The first type of aggregation is mediated through attractive intermolecular interactions between molecules of native structure, in particular at higher protein concentrations (the so-called colloidal aggregation) [11]. This mode of aggregation is often observed among antibodies and is a cause of their anomalous viscosity [12]. The aggregation of antibodies with native structure can be suppressed by chaotropes that reduce the attractive interactions [13]. The second type of aggregation is mediated by partial or extensive changes in the conformation caused by various stresses, e.g., elevated temperature, addition of destabilizing solvent additives, and mechanical stresses [14]. This type of aggregation is usually irreversible and can be

suppressed by either stabilizing the native structure against conformational changes [15] or suppressing the intermolecular interaction between the (partially) unfolded proteins [16]. The second type of aggregation can occur, for example, among antibodies during manufacturing processes, e.g., elution from protein A by a low pH buffer [17].

Solvent additives play an important role in suppressing protein aggregation [18,19]. Various types of solvent additives that either enhance protein stability or suppress protein aggregation have been investigated on globular proteins [18]. The protein stabilizers such as sodium sulfate [20] and sugar [21] interact favorably with water molecules, whereas the protein denaturant destabilizers such as guanidine [22] and urea [23] interact favorably with hydrophobic surfaces of the proteins, reducing the protein–protein interactions. Arginine has the unique property of suppressing protein aggregation without causing denaturation [24–26]. Although the knowledge of a cosolvent has been accumulated [27], it cannot be readily applied for antibodies due to their structural complexity. In this study, I investigated the effects of known protein stabilizers or destabilizers as well as arginine on the heat-induced aggregation of human immunoglobulin (IgG).

3.2. Material and methods

Materials

Human IgG was obtained from MBL life science (Nagoya, Japan). Sodium lauroyl glutamate was kindly provided by Ajinomoto Co., Inc. (Tokyo, Japan). Arginine hydrochloride, sodium chloride, sodium phosphate, sodium thiocyanate (NaSCN), sodium sulfate, guanidine hydrochloride (Gdn), urea, glucose, trehalose dihydrate, xylitol, ethanol, and ethylene glycol were from Wako Pure Chemical Inc., Ltd. (Osaka, Japan). All chemicals used were of reagent grade and used as received. IgG was dissolved in and dialyzed against pure water to remove salts before the following experiments. The protein concentration of the above stock IgG solution was spectrophotometrically determined using a UV-vis spectrophotometer (ND-1000; NanoDrop Technologies, Inc., Wilmington, USA).

Determination of IgG monomer and oligomer concentrations

The stock IgG solution in water was diluted to 2.0 mg/ml sample solution containing solvent additives at the indicated concentration and 100 mM Na-phosphate buffer (pH 7.0). The diluted samples were heated at 75°C for 2 min or longer and cooled at 4°C for 5 min. The samples were then centrifuged at 18,800 *g* for 30 min to remove the insoluble aggregates. After centrifugation, the supernatant was subjected to size exclusion chromatography on a high-performance liquid chromatography (HPLC) using a size exclusion column (Yarra SEC 3000; Phenomenex, Torrance, CA). The column was equilibrated with a running buffer containing 100 mM Na-phosphate buffer (pH 7.0) and 200 mM arginine at a flow rate 1.0 ml/min. A 50 μl aliquot of the supernatants was loaded into the column. The elution was monitored at 280 nm, and the concentration of IgG monomer was calculated from the peak area.

The amount of soluble IgG oligomers was determined as described below. A 10 μl aliquot of the supernatants was diluted with 10 μl of 100 mM Na-phosphate buffer (pH 7.0) containing 8 M Gdn to eliminate the contribution of light scattering of the soluble oligomers to the total absorbance. The absorbance of the mixture was determined using a UV-vis spectrophotometer. This absorbance corresponds to the total protein amount, i.e., the amounts of the monomers and the soluble oligomers. The amount of soluble oligomers was determined by subtracting the monomer amount determined from the size exclusion analysis. The amount of insoluble aggregates was calculated from the total protein subjected to the heat treatment.

Thermal denaturation curve

The thermal denaturation curve of IgG was plotted by near-UV-CD measurement using a J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan) and a 1-cm path-length quartz cell. Briefly, 0.2 mg/ml IgG solution containing the 100 mM Na-phosphate buffer in the presence or absence of 1 M additives was loaded onto the cell. The 2-ml samples in the cell were heated at a rate of 1°C/min increment and monitored at 292 nm.

3.3. Results

IgG at 2.0 mg/ml was heated at 75°C for 2 min in the absence and presence of 500 mM arginine and subjected to size exclusion chromatography. Figure 3.1 shows the chromatogram of the control IgG, i.e., without heating (solid line). The size distribution was identical in the absence (A) and presence (B) of arginine, indicating no effects of arginine on the control sample. Nearly 100% of the loaded IgG was eluted as a monomer at 8–9 min. When heated in the absence of arginine, the monomer peak was greatly reduced (A) and a new peak appeared at 5–6 min corresponding to the soluble IgG oligomers. Figure 3.1A clearly shows that the total peak area was far less than that of the control, consistent with the observed precipitates, and hence, that the heating at 75°C in the absence of arginine resulted in extensive aggregation with a consequent precipitation. In the presence of 500 mM arginine (B), the monomer peak was similarly reduced by the heat treatment, indicating that it does cause protein aggregation. However, the peak area at 5–8 min corresponding to the soluble oligomers was much greater that that observed in the absence of arginine. This is consistent with the few precipitates when heated with arginine than without arginine. Thus, it is evident that while arginine is effective in dispersing heat-generated aggregates into smaller soluble oligomers, it is not effective in preventing the association of monomeric IgG.

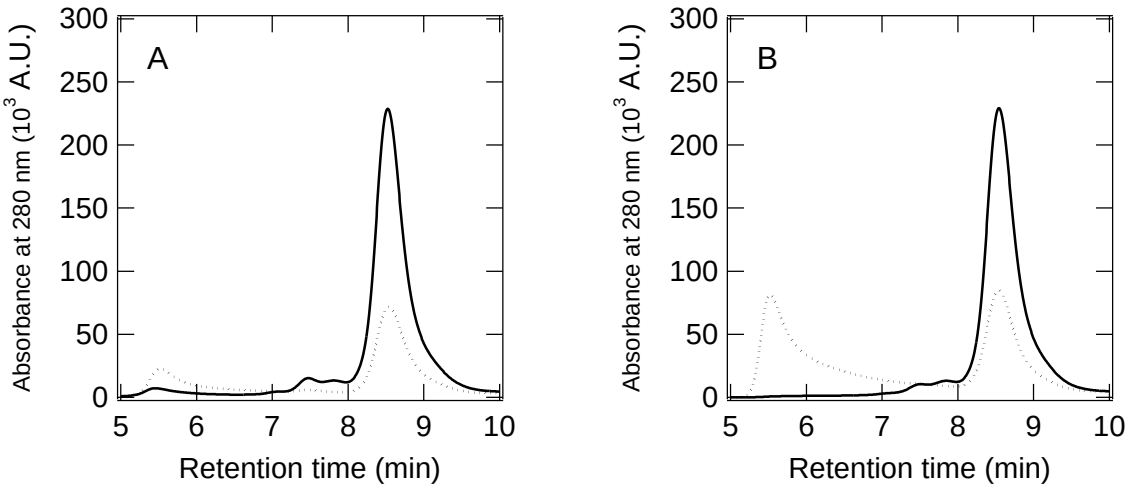

Figure 3.1. Size exclusion chromatography of 2.0 mg/ml IgG solution containing 100 mM Na-phosphate buffer (pH 7.0) in the (A) absence or (B) presence of 500 mM arginine. The sample was heated at 75°C for 0 min (solid line) and 2 min (dotted line).

The effects of various additives on the thermal aggregation of IgG were examined in the presence of 500 mM arginine, i.e., the samples were heated at 75°C for 2 min. Reduction of the monomer content was examined in the absence and presence of 500 mM arginine as a function of incubation time. Figure 3.2. plots the change in the monomer content in the absence (solid circles) and presence (open squares) of arginine. The plots completely overlapped with each other, indicating that the addition of 500 mM arginine showed no impact on the formation of IgG aggregation. The monomer content was reduced to ~25% after heating for 2 min and to ~0% after heating for 20 min regardless of the absence or presence of arginine.

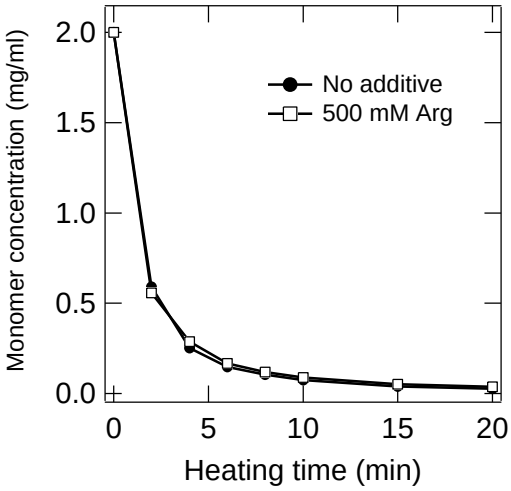

Figure 3.2. Monomer concentration after the heat treatment. IgG solution of 2.0 mg/ml containing 100 mM Na-phosphate buffer (pH 7.0) in the absence (closed circles) or presence (open squares) of 500 mM arginine was heated at 75°C for 0–20 min. The monomer concentration was calculated from the peak of size exclusion chromatography.

The mass balance of IgG after heat treatment is shown in figure 3.3A. While the content of the soluble oligomers (gray bar) was similar to the monomer content (black bar, ~0.5 mg/ml) in the absence of arginine, the content of the soluble oligomers increased 3-fold to ~1.5 mg/ml. As a consequence, there were few precipitates in the presence of arginine (white bar) compared to those with ~0.9 mg/ml insoluble aggregates (left panel of figure 3.3A). One possible explanation is that heat treatment leads to a different protein conformation in the presence of arginine from that in its absence, which still causes a similar IgG association but reduces further aggregation of the soluble oligomers.

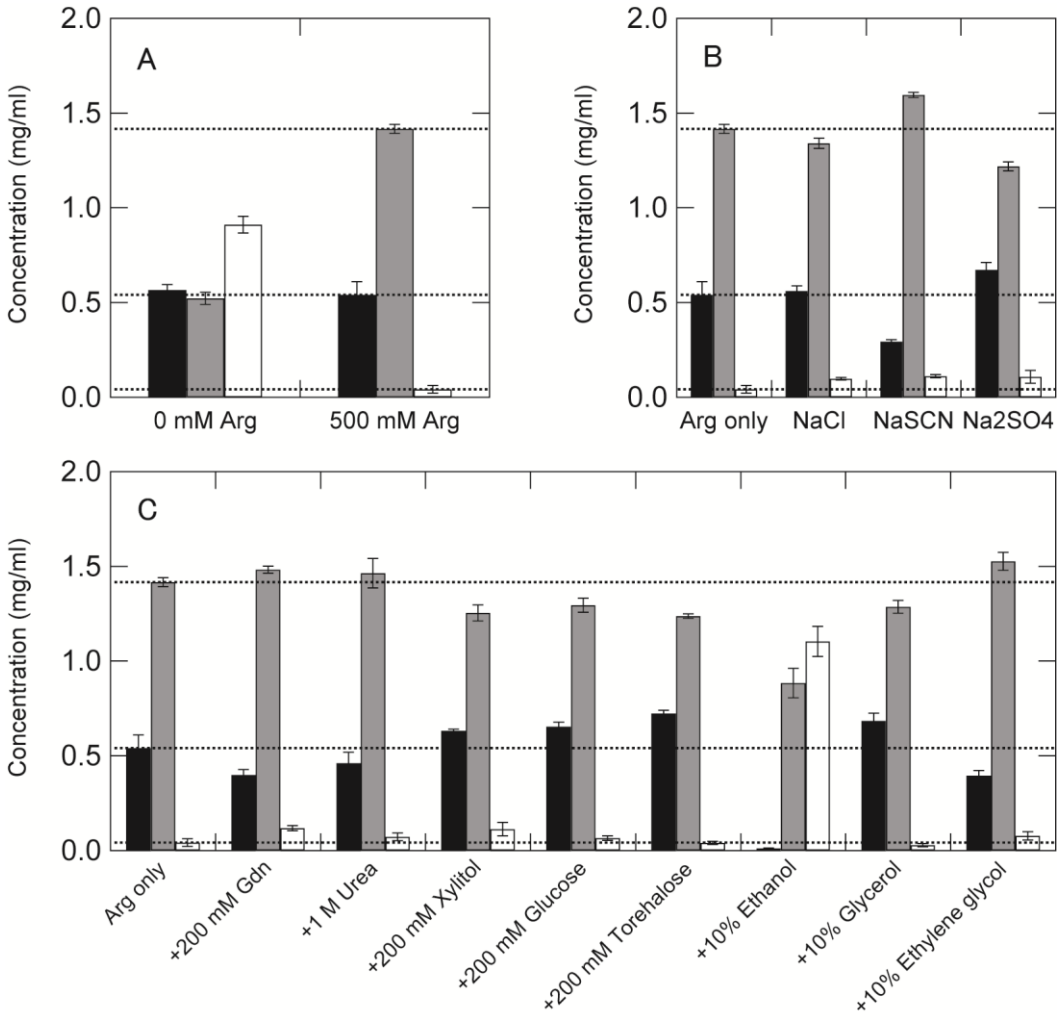

Figure 3.3. Concentration of IgG after the heat treatment. Each solution containing 2.0 mg/ml IgG with various additives (pH 7.0) was heated at 75°C for 2 min, and then the concentrations of the monomer (black), soluble oligomer (gray), and insoluble aggregate (white) of IgG at 2 min were plotted. (A) Comparison of 0 and 500 mM arginine as an additive. (B) Comparison of 500 mM inorganic salts with 500 mM arginine. (C) Comparison of denaturants, osmolytes, and organic compounds with 500 mM arginine. The dotted lines indicate the result in the presence of 500 mM arginine for visual comparison. The experiment was replicated three times, and the error bars indicate the standard deviation.

The distribution of monomers, soluble oligomers, and precipitates was similarly determined by size exclusion chromatography and UV-vis spectrophotometer. Figure 3.3B shows the effects of various salts with 500 mM arginine. It was observed that the addition of these salts to 500 mM arginine solution increased, though slightly, the insoluble aggregates (white bar) relative to the amount with arginine alone, suggesting that the reduction of electrostatic repulsion between IgG may be responsible for the formation of insoluble aggregates during heat treatment. Sodium chloride showed no change in the monomer content (black bar),

decreased slightly the soluble aggregates (gray), and increased the precipitates (white). This may be due to a weak salting-out effect of this salt, leading to small-enhanced aggregation. NaSCN, known as a salting-in salt, further decreased the monomer content and increased both soluble and insoluble aggregate contents. Thus, this salt appeared to enhance the aggregation of monomeric IgG and thereby further reduce the monomer content. This may be due to the destabilizing effects of NaSCN. Interestingly, sodium sulfate (Na_2SO_4), a known salting-out salt, increased the monomer content, which is in contrast to the effects of NaSCN. It is likely that this salt stabilized the IgG structure against heat denaturation and thereby prevented IgG from monomeric association of the heat-denatured state. However, it acted as a salting-out salt on soluble oligomers, as it further decreased the soluble oligomers and increased the insoluble aggregates.

Figure 3.3C shows the results of the effects of various additives at the indicated concentration in the presence of 500 mM arginine after 2-min heat treatment. Gdn even at 200 mM was sufficient to further reduce the monomer content, similar to the results with NaSCN. Moreover, similar to NaSCN, 200 mM Gdn enhanced the formation of both soluble oligomers and insoluble aggregates. Urea at 1 M showed a similar trend to that of 200 mM Gdn and 500 mM NaSCN. Sugars (glucose and trehalose) and polyhydric alcohol (xylitol) at 200 mM showed a similar trend with each other, increasing the monomer content and decreasing the soluble oligomer content, which were similar to the effects of NaCl and Na_2SO_4. Thus, their effects may be explained by stabilization of the protein structure against heat denaturation [18,28]. Ethanol at 10% resulted in no remaining monomers with the formation of both soluble oligomers and insoluble aggregates. This is most likely due to enhanced denaturation of IgG by ethanol even at 10%. Additional polyhydric alcohols (glycerol and ethylene glycol) at 10% resulted in opposite effects. Glycerol increased the monomer content due to its stabilizing effects [29], whereas ethylene glycol reduced the monomer content due to its destabilizing effects. A similar result was reported using lysozyme [30]. Both are polyhydric alcohols, but they behaved differently, i.e., ethylene glycol as a destabilizing additive and glycerol as a stabilizing additive. Thus, it does appear that the effects of the polyhydric alcohols on the stability are correlated with the number of OH group [31].

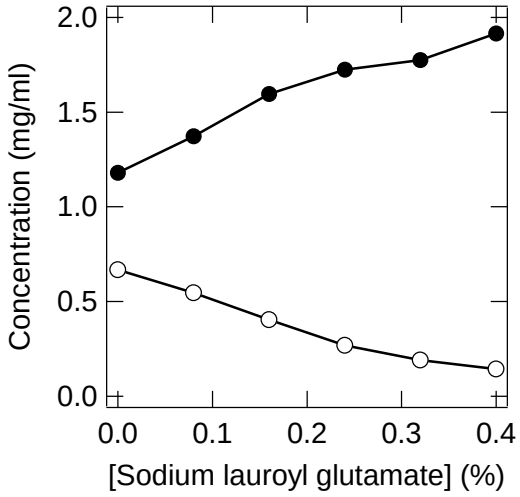

Figure 3.4. Concentration of monomer (open circles) and soluble aggregate (closed circles) of IgG solution containing 0%–0.4% sodium lauroyl glutamate with 500 mM arginine after the heat treatment at 75°C for 2 min.

It has been shown that sodium lauroyl glutamate is a mild detergent and can solubilize insoluble proteins [32]. Figure 3.4 shows the monomer and soluble oligomer contents of IgG as a function of lauryl glutamate concentration when heated at 75°C for 2 min in the presence of 500 mM arginine. As the concentration of lauryl glutamate was increased, the monomer content gradually decreased (white circles) concomitant with increasing soluble oligomer content (solid circles). There were few monomers left with 0.4% lauroyl glutamate due to its denaturing effects.

The results described above indicated that the stabilizing additives increased the monomer content in the presence of 500 mM arginine. Their effects were then examined in the absence of arginine and compared with the results in the presence of 500 mM arginine. Figure 3.5A shows the effects of 0–1000 mM additives on the monomer content after heating at 75°C for 2 min. The monomer content was independent of the arginine concentration and reduced to ~30% of the total protein (closed circles). Glycerol, which increased the monomer content when heated in the presence of 500 mM arginine, was ineffective by itself regardless of the concentration (closed triangles). On the contrary, trehalose was highly effective in increasing the monomer content, which nearly linearly increased with the concentration (open squares), consistent with its effects in the presence of 500 mM arginine. This is most likely due to its effectiveness as a protein stabilizer. In fact, glucose (open circles), xylitol (closed squares), and sodium sulfate (open triangles) showed a more or less

similar trend. All of them increased the monomer content, the magnitude of which was between those of trehalose and arginine/glycerol. Figure 3.5B shows the results of the soluble oligomer content. Arginine was most effective in increasing the soluble aggregates (closed circles). Sodium sulfate showed little effects on the soluble oligomer content up to 500 mM (open triangles). However, this salt at 750 mM, despite increasing the monomer content, resulted in reduction of soluble aggregates. In fact, 1000 mM sodium sulfate resulted in spontaneous precipitation at room temperature even before heating; no data were obtained at 1000 mM sodium sulfate as shown in figure 3.5. Glycerol, which showed no effects on the monomer content, resulted in a linear increase in the soluble oligomer content (close triangles). Trehalose (open squares), which showed a linear increase in the monomer content, showed a bell-shaped concentration dependence. It increased the soluble oligomers at 250 mM followed by a gradual reduction. Glucose (open circles) and xylitol (close squares) showed a more or less similar trend, slightly increasing the soluble oligomers.

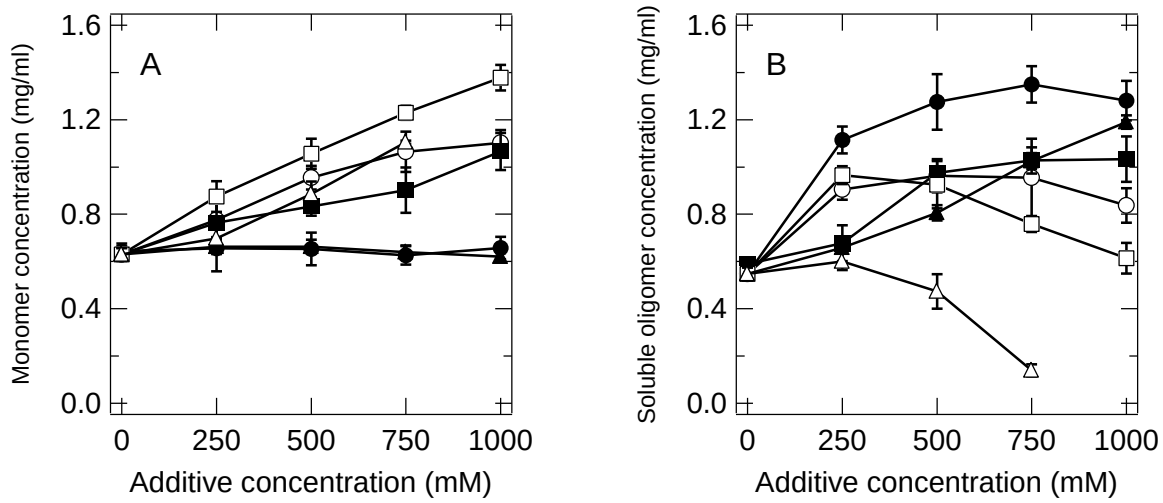

Figure 3.5. Concentration of monomer (A) and soluble oligomer (B) of IgG after the heat treatment at 75°C for 2 min. The solutions contained 2.0 mg/ml IgG, arginine (closed circles), glucose (open circles), xylitol (closed squares), trehalose (open squares), glycerol (closed triangles), and sodium sulfate (open triangles). The measurements were performed three times, and the error bars depict the standard deviation of the mean.

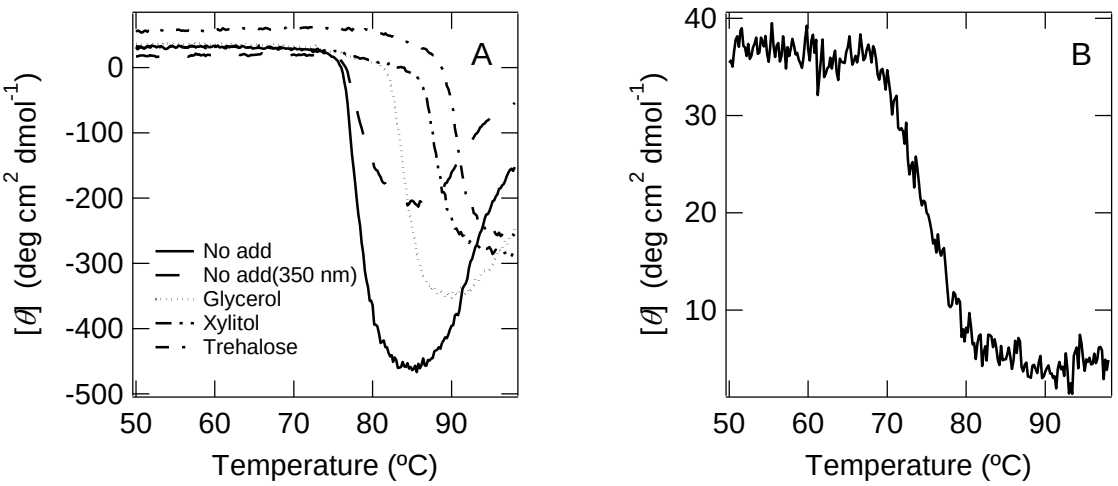

Figure 3.6. Thermal denaturation curve of 0.2 mg/ml IgG in the presence or absence of additives. (A) No additive (solid line), no additive measured at 350 nm (dashed line), 1000 mM glycerol (dotted line), 1000 mM trehalose (single dotted chain line), 1000 mM xylitol (double dotted chain line). (B) 1000 mM Arg. The circular dichroism was measured at 292 nm.

Next, thermal denaturation experiments were carried out to assess whether some of the structure-stabilizing additives at 1000 mM in fact stabilized the IgG used in this study. Figure 3.6 shows the change in ellipticity monitored at 292 nm with increasing temperature. For the control (without additive), the CD intensity sharply decreased at above ~75°C, indicating that the temperature used to induce aggregation was at the edge of thermal melting of the IgG. This temperature increased in the order of glycerol < xylitol < trehalose and decreased to ~70°C with arginine. It is noticeable that the CD intensity at 292 nm did not decrease to zero and instead became extremely negative, suggesting a possibility that the thermal denaturation induced a new tertiary structure with a negative CD intensity at 292 nm. However, when monitored at 350 nm, the CD intensity still went to negative. Since there is little absorbance at 350 nm for IgG or any proteins, this negative CD is not due to the formation of new structures but must be due to light scattering.

3.4. Discussion

In this study, I have investigated the effects of various additives on the thermal aggregation of IgG. Mechanistic understanding of the observed thermal aggregation and the effects of each solvent additive may be explained using a scheme depicted in figure 3.7, in which the aggregation is shown to be mediated by conformational changes at 75°C. It is unlikely that the observed results involve reversible colloidal aggregation in such a low IgG concentration of 2.0 mg/ml. In addition, the IgG samples were heated at 75°C, which facilitates the unfolding of IgG. Based on the reported weaker stability of Fab domain [4], it is likely that this domain first unfolds (i), which triggers the formation of soluble oligomers (ii). Further heating causes additional unfolding (iii), likely on a more stable Fc domain, leading to the last step (iv), i.e., aggregation via the Fc domain. The formation of soluble oligomers was shown to occur by other stresses such as acid exposure [33], agitation [34,35], and lyophilization [36]. Thus, attention should be paid on the formation of soluble oligomers in the process of immunoglobulin aggregation.

The additives used in this study showed differential effects on the immunoglobulin aggregation (Figure 3.2). The most intriguing observation is that arginine showed no effects on the amount of monomers. Namely, arginine was unable to preserve the monomer content against heat treatment. However, it did suppress the formation of insoluble aggregates (Figure 3.3A). Based on the thermal unfolding data (Figure 3.6B), arginine may facilitate partial unfolding (i), as it lowers, though slightly (a less degree), the melting temperature. Nevertheless, arginine showed no impact on the monomer content, indicating the competition between facilitation of unfolding and the suppression effects of aggregation. The observed stabilization of soluble oligomers (Figure 3.3A) should be imposed on the next steps (iii) and (iv). Considering the slight destabilizing effects of arginine, it is highly unlikely that arginine inhibits step (iii). Therefore, arginine should inhibit the last step (iv). It is interesting that while arginine cannot inhibit the intermolecular interaction between Fab domains (ii), it can inhibit the other intermolecular interaction between Fc domains (iv). Arginine has been suggested to suppress weak molecular interactions, which in turn suggests that the molecular interaction in (ii) may be stronger than that in (iv). A similar trend was observed with a monoclonal antibody, which was subjected to mild heat stress at low pH in the absence and presence of arginine at high concentration (personal

communication). Arginine was ineffective in preserving the monomeric antibody but was effective in preserving the soluble oligomers against the above treatment.

How about other additives? The thermal denaturation curve results showed that the denaturation can be suppressed by xylitol, trehalose, and glycerol. The observed increase in the monomer content by trehalose and xylitol as well as glucose and sodium sulfate may be explained by the stabilization of the native monomer. However, no stabilization of the monomers by glycerol alone suggests the involvement of other mechanisms. Their effects, except for sodium sulfate, on the soluble oligomers are complex and may be due to a balance between their structure stabilization effects and enhancement of protein–protein interactions. The effect of sodium sulfate is due to its overwhelming salting-out effects on the unfolded IgG, leading to the formation of insoluble aggregates or precipitates.

In summary, this study demonstrated the pathway of heat-induced aggregation of IgG and the effects of aggregation suppressors or protein stabilizers. Unlike the globular protein, the thermal aggregation pathway of IgG shows multiple steps, including the soluble oligomer state. The selection of additives strongly influences each process depending on its mechanisms. This study will be useful for the rational formulation design of immunoglobulins.

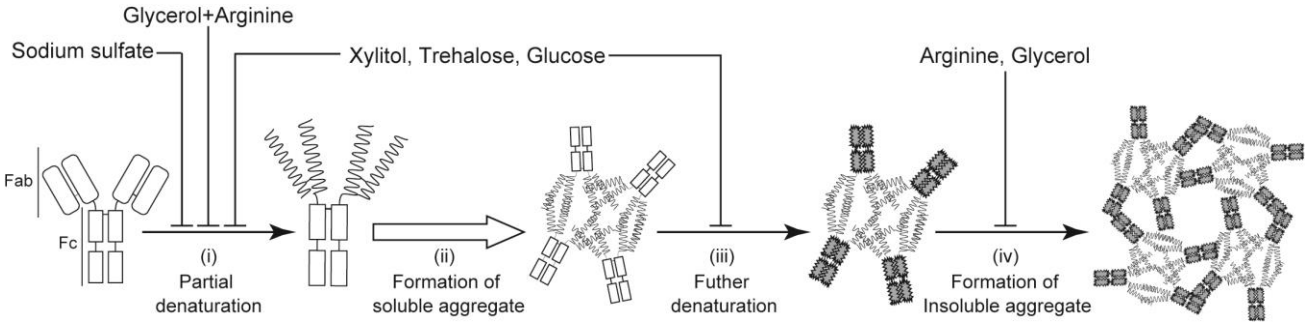

Figure 3.7. Schematic illustration of the aggregation of IgG and the effect of additives.

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Chapter 4.

Trimethylamine N-oxide (TMAO) is a Counteracting Solute of Benzyl Alcohol for Multi-Dose Formulation of Immunoglobulin

4.1. Introduction

Immunoglobulins are the important class of biopharmaceuticals [1–3]. However, immunoglobulins are expensive due to high manufacturing cost [4–6]. For example, the cost of a monoclonal antibody is usually about the order of $10,000 per year [7], meaning that any development to reduce the cost is of great importance [8]. Multi-dose formulation is a liquid vial containing more than the amount of a single injection, for example as in an eye lotion [9] and has some advantages, including less product wastage and lower packing cost, over the single dose formulation. Thus, multi-dose vials should be more economical [10,11]. In fact, some biopharmaceuticals, including insulin, are available as multi-dose formulations. A multi-dose formulation requires the addition of an antimicrobial preservative to inhibit the growth of bacteria during the storage period [12]. It has been reported that some antimicrobial preservatives induced protein aggregation [13–21] due to their hydrophobic properties and denaturing effects [19]. Thus, any development to reduce protein aggregation induced by the preservatives is of great significance.

Using a cosolvent is a simple method to control protein aggregation. Till date, several additives have been developed to suppress the aggregation of biopharmaceuticals [22,23]. The mechanism of aggregation suppression depends on the types of additives [24]. Briefly, protein stabilizers such as trehalose are preferentially excluded from the protein surface, leading to enhancement of structural stability [25]. In contrast, protein denaturants such as guanidine hydrochloride [26] and urea [27] interact favorably with the hydrophobic protein surface, weakening both intra- and inter-molecular interactions. Unlike these cosolvents, arginine has a unique property, by which it interacts with the aromatic surface of the protein via pi-cation interaction without

causing protein denaturation and thereby prevents protein aggregation [28,29]. Thus, it may be possible that the cosolvent can prevent the preservative-induced aggregation of biopharmaceuticals. In this study, I demonstrate the effects of a cosolvent on the benzyl alcohol-induced aggregation of human immunoglobulin G (IgG) as a model system for multi-dose formulation.

4.2. Material and methods

Materials

Human IgG was obtained from MBL life science (Nagoya, Japan). L-Arginine ethyl ester dihydrochloride and trimethylamine N-oxide (TMAO) dehydrate were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Benzyl alcohol, sodium dihydrogen phosphate, arginine hydrochloride, glucose, trehalose dehydrate, and xylitol were from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All chemicals used were of reagent grade and used as received. IgG was dissolved in and dialyzed against pure water to remove salts before the following experiments. The protein concentration of the above stock IgG solution was spectrophotometrically determined using a UV-vis spectrophotometer (ND-1000; NanoDrop Technologies, Inc., Wilmington, USA).

Determination of the amount of IgG aggregate

The stock IgG solution in water was diluted to 2.0 mg/ml in the presence of solvent additives at the indicated concentration and 100 mM Na-phosphate buffer (pH 7.0). The diluted samples were heated at 40°C or 45°C for the indicated time and cooled at 4°C for 5 min. The samples were then centrifuged at 18,800 g for 20 min to remove the insoluble aggregates. After centrifugation, the supernatant was subjected to size exclusion chromatography on a high-performance liquid chromatography (HPLC) using a size exclusion column (Yarra SEC 3000; Phenomenex, Torrance, CA). The column was equilibrated with a running buffer containing 100 mM Na-phosphate buffer (pH 7.0) and 200 mM arginine at a flow rate 1.0 ml/min. A 50 µl aliquot of the supernatant was loaded into the column. The elution was monitored at 280 nm, and the concentration of native IgG was calculated from the peak area. The amount of IgG aggregate was determined from the reduction of the native IgG.

Thermal denaturation curve measurement

The thermal denaturation curve of IgG was obtained by near-UV CD measurement using a J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan) and a 2-mm path length quartz cell. Briefly, 2.0 mg/ml IgG solution containing the 100 mM Na-phosphate buffer (pH 7.0) in the presence or absence of 500 mM additives was loaded onto the cell. Then, 800 µl of the samples in the cell were heated at an increment rate of 1°C/min and monitored at 292 nm.

Solubility of benzyl alcohol

The solubility of benzyl alcohol at pH 7.0 was determined in the absence and presence of 0–1,000 mM additives. Stock solutions of the additives at 0–1,000 mM were prepared in 100 mM Na-phosphate buffer (pH 7.0). An excess amount of benzyl alcohol was added into the stock aqueous additive solutions. Benzyl alcohol was completely dissolved in the test solvents in boiling water and then cooled to 25°C and incubated at this temperature for 1 h to reach equilibrium solubility. After the incubation, the samples were centrifuged at 18,800 g for 20 min. The supernatant of the samples was diluted 10-fold with distilled water and then measured for benzyl alcohol concentration by absorbance at 256 nm. The solubility was then calculated from the standard curve.

4.3. Results

First, I determined the experimental condition to examine the effects of benzyl alcohol on protein aggregation. The concentration of benzyl alcohol used was 2%, which is the maximum concentration for the multi-dose vials [12]. A temperature range of 40°C–45°C is used for the accelerated stability test [30], which is below the commencement of thermal unfolding of the Fab fragment, i.e., 50°C in the absence of benzyl alcohol [31]. The 2.0 mg/ml IgG was heated at 45°C for 10 min in the presence or absence of 2% benzyl alcohol. Figure 4.1A shows the chromatogram of IgG without benzyl alcohol. Before the heat treatment, two peaks were obtained, i.e., a main peak at 8–9 min and a small peak at around 7 min, corresponding to the monomer and dimer of IgG. After the heat treatment, the chromatogram was identical to that before the heat treatment, indicating no effects of heating on IgG aggregation in the absence of benzyl alcohol. Figure 4.1B shows the effects of 2%

benzyl alcohol on IgG solution. The chromatogram of IgG before heating was identical to the results in the absence of benzyl alcohol, indicating no effects of benzyl alcohol at 45°C. After the heat treatment, a new peak appeared at 5 min, corresponding to the soluble oligomers. It is thus clear that benzyl alcohol induces formation of soluble aggregates when heated at 45°C.

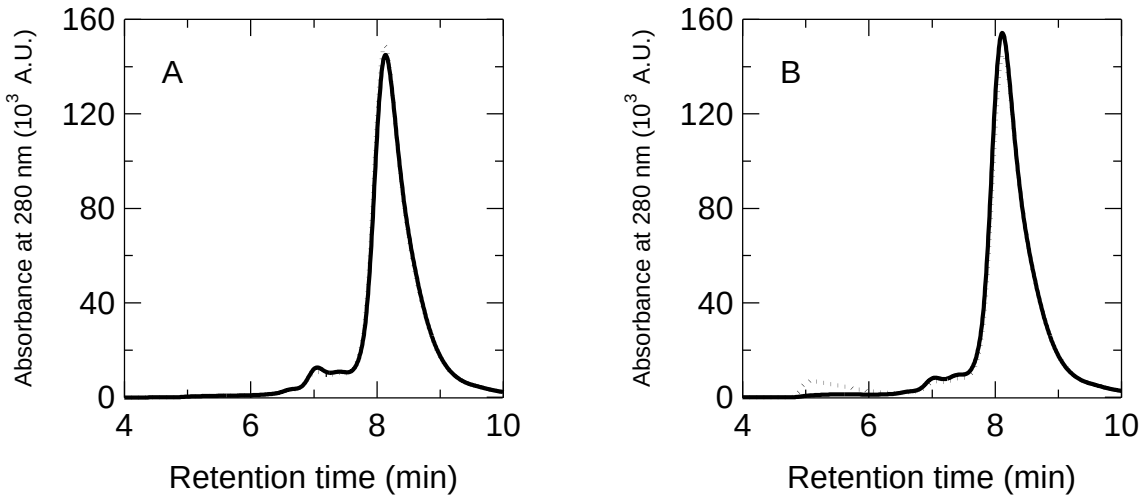

Figure 4.1. Size exclusion chromatogram of 2.0 mg/ml IgG solution containing 100 mM Na-phosphate buffer (pH 7.0) in the absence (A) or presence (B) of 2% benzyl alcohol. The sample was heated at 45°C for 0 min (solid line) and 10 min (dotted line).

I next examined the effects of various additives under the similar conditions. Figure 4.2 shows the amounts of IgG aggregates in 2% benzyl alcohol after the heat treatment at 45°C for 10 min in the presence of various additives. In the absence of benzyl alcohol, a small amount of IgG aggregates was observed after the heat treatment. In the presence of 2% benzyl alcohol, heat treatment resulted in approximately 5% aggregation. I then compared a few protein stabilizers, trehalose, xylitol, and glucose [32] at 500 mM. The results obtained were somewhat unexpected. Trehalose reduced IgG aggregation to 4%. Glucose significantly increased the aggregate content to 7%. Xylitol showed no influence on IgG aggregation. These results are not fully consistent with the general stabilization effects of sugars and polyols. Arginine and its ethyl ester derivative, which have been reported as superior aggregation suppressors [29,33], were also tested. On the contrary to the expectation, these additives, in particular arginine ethyl ester, facilitated the aggregation of IgG. TMAO, known as a counteracting osmolyte against the denaturation effect of urea [34–36], decreased the aggregation to 4%.

Caprylate at 20 mM facilitated the aggregation of IgG, although it inhibited the aggregation of bovine gamma globulin [37]. Altogether, these experiments showed that trehalose and TMAO are effective agents against benzyl alcohol-induced IgG aggregation.

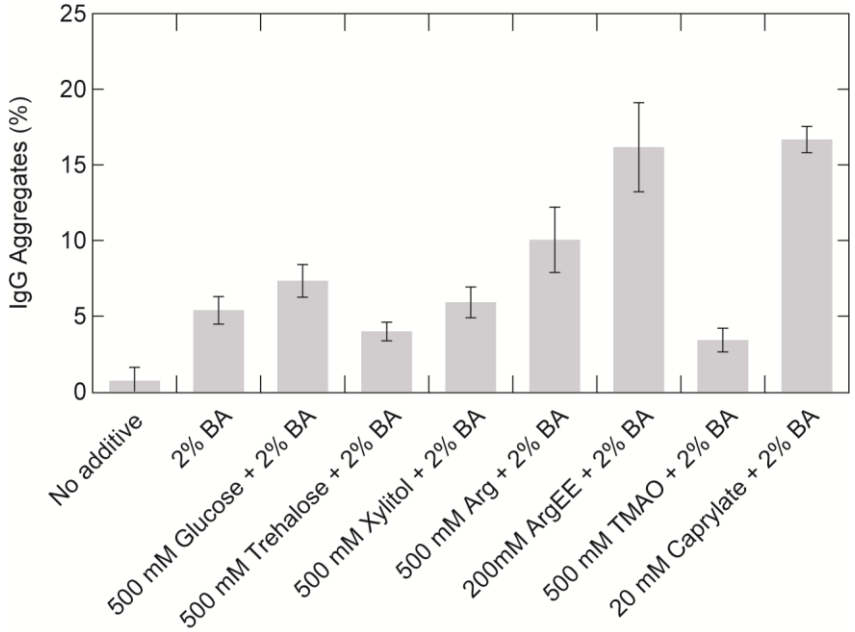

Figure 4.2. Effect of additives on the amount of aggregates induced by benzyl alcohol (BA). Sample solutions containing 2.0 mg/ml IgG and various additives were heated at 45°C for 10 min. Arg, arginine; ArgEE, arginine ethylester; BA, benzyl alcohol. The experiments were replicated three times, and the error bars indicate the standard deviation.

Next, I investigated the effects of trehalose and TMAO on long-term storage of IgG in 2% benzyl alcohol solution. Figure 4.3 shows the amount of IgG aggregates after incubation for 1 day and 7 days at 40°C. In the absence of benzyl alcohol, a negligible amount of IgG aggregates was formed after storage, indicating that IgG is fairly stable at 40°C. The amounts of IgG aggregates in the presence of 2% benzyl alcohol reached 10 and 22% after 1 day and 7 days of storage. TMAO mitigated the effect of benzyl alcohol and reduced the amounts of IgG aggregates to 5 and 10% after 1 day and 7 days of storage. In contrast, trehalose did not suppress the aggregation of IgG, reaching 23% after 7 days of storage, while it was slightly effects on short-term incubation. These data indicate that TMAO is the best additive for long-term storage of IgG in the presence of benzyl alcohol.

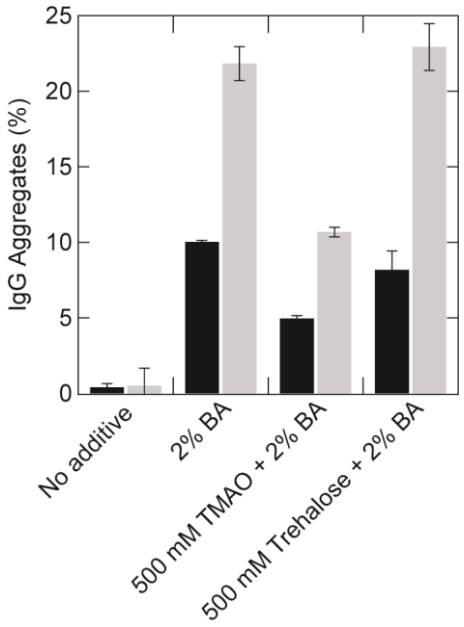

Figure 4.3. Effect of TMAO and trehalose on the amount of IgG aggregates induced by benzyl alcohol (BA). Sample solutions containing 2.0 mg/ml IgG and additives were heated at 40°C for 1 day (black bars) or 7 days (gray bars). The experiments were replicated three times, and the error bars indicate the standard deviation.

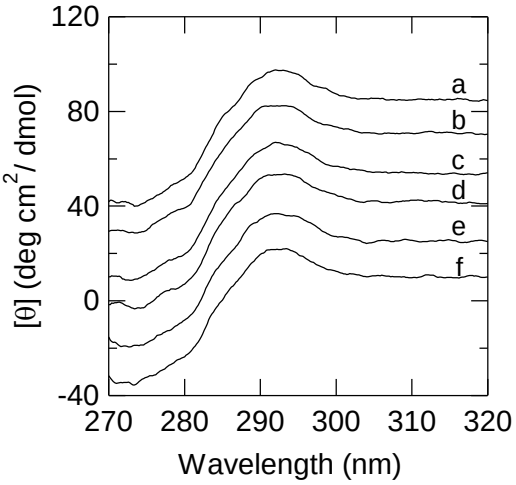

Figure 4.4. Near-UV CD spectra of IgG in the presence of various additives. Solutions containing 2.0 mg/ml IgG in the absence of additives (a) or the presence of 2% benzyl alcohol (b), 500 mM TMAO (c), 500 mM xylitol (d), 500 mM trehalose (e), and 500 mM glucose (f). Each spectrum was shifted upward by 15 deg cm^2/dmol for visual comparison.

Near UV-CD spectra were measured to obtain information about the tertiary structure of IgG in various solutions (Figure 4.4). The spectrum of IgG in the absence of additives showed a positive peak at around 292 nm, which is consistent with the previous study [38]. The spectrum of IgG did not change in the presence of 2% benzyl alcohol or 500 mM additives, indicating that neither benzyl alcohol nor the additives influenced the tertiary structure at 25°C.

To examine the thermal stability of IgG, the change in ellipticity at 292 nm was measured as a function of temperature (Figure 4.5). In the absence of additives, the ellipticity sharply decreased at 75°C, as shown in figure 4.5A. The addition of 2% benzyl alcohol greatly lowered the thermal melting temperature of IgG to 60°C, indicating that benzyl alcohol significantly destabilized the tertiary structure of IgG. Figure 4.5B shows the effects of 500 mM TMAO, glucose, xylitol, and trehalose additives on the stability of IgG in the presence of 2% benzyl alcohol. These additives increased the melting temperature of IgG in the above order, at least partially restoring the decreased melting temperature by 2% benzyl alcohol. It was unexpected that TMAO was less effective in increasing the melting temperature of IgG in the presence of benzyl alcohol than the other additives. If the aggregation suppression of TMAO is caused by the structural stabilization, TMAO should be more effective for stability of IgG than other additives. Thus, these data indicate that the stabilization of protein structure by TMAO in benzyl alcohol solution cannot fully explain its aggregation suppression effect.

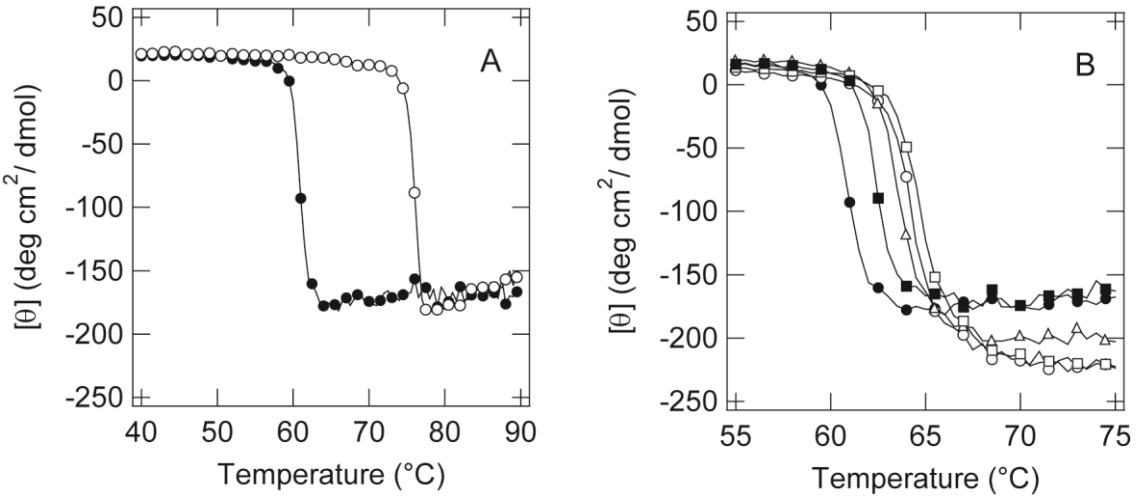

Figure 4.5. Thermal denaturation curve of 2.0 mg/ml IgG in the presence or absence of additives. (A) No additive (open circles) and 2% benzyl alcohol (closed circles). (B) 2% benzyl alcohol (closed circles), 2% benzyl alcohol and 500 mM TMAO (closed squares), 2% benzyl alcohol and 500 mM glucose (open triangles), 2% benzyl alcohol and 500 mM xylitol (open circles), and 2% benzyl alcohol and 500 mM trehalose (open squares).

The above results show that there appears to be another mechanism of TMAO as an aggregation suppressor on top of its structure stabilization of IgG. While the structure stabilization of TMAO may arise from preferential exclusion of TMAO from the protein surface, the aggregation suppression effect may also arise from the direct interaction between TMAO and benzyl alcohol. To investigate the interaction between benzyl alcohol and the additives, the solubility of benzyl alcohol was examined in the presence of TMAO and trehalose. Figure 4.6 shows that in the absence of the additives, the solubility of benzyl alcohol was about 360 mM at pH 7.0, which is consistent with the previous study [39]. The solubility of benzyl alcohol slightly but monotonically increased with increasing concentrations of TMAO, indicating that TMAO interacts with benzyl alcohol. In contrast, trehalose decreased the solubility of benzyl alcohol, consistent with its highly hydrophilic properties. TMAO has methyl groups, providing significant hydrophobicity and thereby potential binding sites for benzyl alcohol. The results suggest that the interaction of TMAO with benzyl alcohol plays an important role in suppression of benzyl alcohol-induced aggregation of IgG.

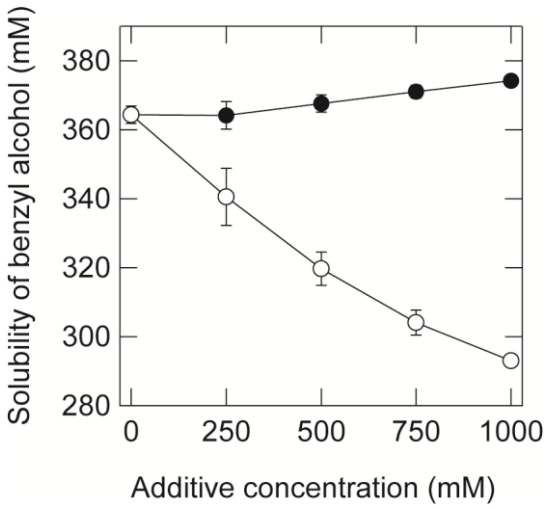

Figure 4.6. Solubility of benzyl alcohol in the presence of TMAO (closed circles) and trehalose (open circles). All solutions contained 100 mM Na-phosphate buffer at pH 7.0.

4.4. Discussion

In this study, I examined the effect of various additives on the aggregation of IgG induced by moderate temperature and benzyl alcohol. I found that TMAO shows a positive effect against the benzyl alcohol-induced aggregation during long-term storage. The molecular mechanism by which TMAO suppresses the benzyl alcohol-induced aggregation of IgG appears to be somewhat complex. First, the previous study has shown that a protein stabilizer, typically sucrose, minimizes the amount of antimicrobial-induced aggregation of human interleukin-1-receptor antagonist [16] and human granulocyte colony-stimulating factor [17]. In addition, the previous study showed that sugars prevent the heat-induced aggregation of IgG [32]. This is because sugars stabilize the tertiary structure of IgG. However, the present study showed that sugars are not effective in suppressing the benzyl alcohol-induced aggregation of IgG. This is perhaps because benzyl alcohol-induced aggregation of IgG is caused by a mechanism different from the mechanism operating in heat-induced aggregation as described below (Figure 4.7).

The aggregation mechanism of IgG is generally described by two steps, i.e., denaturation and aggregation [32]. The first step occurs due to various stresses such as heating [32], agitation [40], and acid exposure [41]. The second step is mediated via intermolecular interactions, including hydrophobic and electrostatic interactions [22]. Thus, the method to prevent the protein from aggregation is to suppress either or both of these steps. To explain this point more clearly, thermal stress unfolds the IgG structure, leading to the formation of soluble oligomers (Figure 4.7A). The formation of soluble oligomers of IgG by heat does not appear to be effectively prevented by arginine or a surfactant [32]. Thus, it is necessary to inhibit the thermal denaturation step in order to prevent IgG aggregation. According to results obtained with interferon-γ [15] and human interleukin-1 receptor antagonist [16], it is believed that benzyl alcohol facilitates the partial unfolding of IgG via hydrophobic interaction with the hydrophobic site of the protein, leading to the formation of aggregates. Therefore, the strategy to minimize the benzyl alcohol-induced aggregation of IgG is (i) prevention of the interaction between benzyl alcohol and IgG and (ii) stabilization of the aggregation prone region against the environmental stresses.

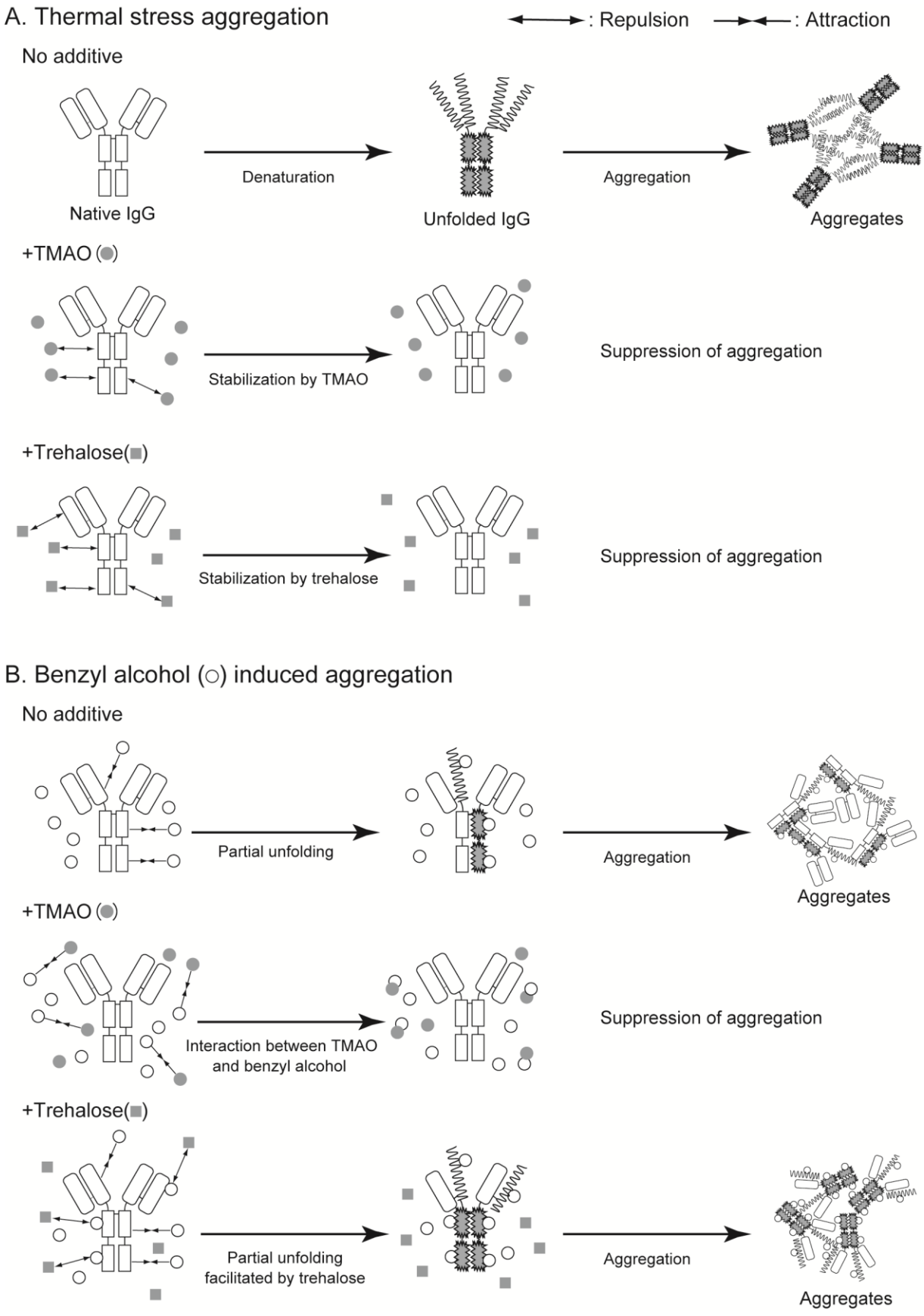
A. Thermal stress aggregation
: Repulsion
: Attraction
No additive
Native IgG
Denaturation
Unfolded IgG
Aggregation
Aggregates
+TMAO (●)
Stabilization by TMAO
Suppression of aggregation
+Trehalose(■)
Stabilization by trehalose
Suppression of aggregation
B. Benzyl alcohol (○) induced aggregation
No additive
Partial unfolding
Aggregation
Aggregates
+TMAO (●)
Interaction between TMAO
and benzyl alcohol
Suppression of aggregation
+Trehalose(■)
Partial unfolding
facilitated by trehalose
Aggregation
Aggregates

Figure 4.7. (Previous page). Mechanism of IgG aggregation by benzyl alcohol. (A) In the thermal stress condition, both TMAO and trehalose prevent the aggregation of IgG with their preferential exclusion mechanism. (B) In the presence of benzyl alcohol, TMAO suppresses the interaction between benzyl alcohol and IgG due to its hydrophobic interaction, leading to prevention of the partial unfolding. On the contrary, trehalose facilitates the interaction between benzyl alcohol and IgG due to the unfavorable interaction between trehalose and benzyl alcohol, leading to enhancement of unfolding stress.

Small molecular additives have been used to stabilize the proteins against thermal stresses and prevent aggregation [24,35]. TMAO is a naturally occurring osmolyte that counteracts the destabilizing effects of urea [34]. The primary mechanism of the stabilization effect of TMAO is the preferential exclusion of TMAO from the protein surface [36] (Figure 4.7A). Moreover, TMAO has three nonpolar methyl groups that weaken the hydrophobic interaction between naphthalene and urea [42]. In fact, TMAO increases the solubility of benzyl alcohol, indicating the presence of a favorable interaction between TMAO and benzyl alcohol (Figure 4.6). Thus, it is possible that the hydrophobic interaction between IgG and benzyl alcohol could be inhibited by TMAO, leading to the reduction in hydrophobic binding of benzyl alcohol to IgG and the amount of IgG aggregates (Figure 4.7B). In contrast, trehalose interacts unfavorably with benzyl alcohol, which may enhance the interaction between benzyl alcohol and IgG. Therefore, the enhanced interaction between benzyl alcohol and IgG by this sugar may counteract its stabilizing effect on protein during long-term storage at 40°C, although the overall tertiary structure of IgG is stabilized by trehalose. It has been reported that trehalose has little effect on IgG aggregation even at 45°C, because IgG has higher resistance against denaturation below 50°C[31]. Thus, it is possible that trehalose may inhibit the heat-induced aggregation of IgG. Other additives, arginine, arginine ethyl ester [33,43,44], and caprylate [37], are known to function as aggregation suppressors due to the weak hydrophobic interaction but decrease the protein stability. Thus, these additives rather enhance denaturation of IgG by benzyl alcohol, leading to no or adverse effects on benzyl alcohol-induced aggregation (Figure 4.2). Taken together, these results suggest that two properties are needed to inhibit the benzyl alcohol-induced aggregation, i.e., the stabilizing effect and the hydrophobic interaction.

In summary, this study showed that TMAO can be used as a protective agent against the benzyl alcohol-induced aggregation of IgG due to its stabilizing effect on protein structure and the hydrophobic interaction between benzyl alcohol and TMAO. Other antimicrobial preservatives such as *m*-cresol and phenol

have a similar structure to that of benzyl alcohol. Thus, it is possible that TMAO is effective against the aggregation by other preservatives. It has been reported that TMAO can be efficiently removed by the normal kidney [45], and hence it is proposed that TMAO is one of the best counteracting solutes against benzyl alcohol for medical applications of proteins. This information will be useful for the application of multi-dose vial formulation of monoclonal antibodies.

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Chapter 5.

Dependence of Ethanol Effects on Protein Charges

5.1. Introduction

Ethanol is routinely used as a disinfectant [1–3]. As it is highly effective against enveloped viruses, the effects of ethanol are due to disruption of membrane structures, leading to virus inactivation [4,5]. Such effects are explained in terms of hydrophobic properties of ethanol [6]. However, ethanol can also inactivate non-enveloped viruses pH dependently [7], although at higher concentrations than normally required for enveloped viruses [5], indicating that disruption of membrane structure may not be a sole factor responsible for virus inactivation by ethanol. One possible mechanism of such inactivation by ethanol is its effects on proteins. Ethanol affects proteins in aqueous solution. It can denature proteins [8–11], often accompanied by transition in secondary structure [12,13] and reduce their solubilities [14,15]. Thus, ethanol effects on virus infectivity could be due to pH-dependent denaturation or altered protein-protein interactions of virus proteins by ethanol. Virion structure of viruses is composed of major coat proteins and spike proteins. Viruses have been shown to have widely different isoelectric points [16], reflecting in part different charged states of these viral proteins [17]. Such differences in charged state may affect the sensitivity to ethanol, as ethanol can alter the free energy of charged groups [6]. The structure analysis of viral proteins at different pH values and ethanol concentration would reveal potential mechanism of virus inactivation by ethanol, though accompanied by great difficulty. Alternatively, I have investigated the effects of ethanol on the structure and aggregation of two model proteins, bovine serum albumin (BSA) and ribonuclease A (RNase A), at pH 4.0 and 7.0. These pH values were chosen to alter the charged state of the proteins. BSA is oppositely charged at these pH values [18], while RNase A is positively charged at different degrees [19]. Such studies should show potential impact of pH and ethanol that might simulate the structure changes or changes in inter-molecular interactions of viral proteins.

5.2. Material and methods

Materials

Anhydrous citric acid, trisodium citrate, sodium dihydrogen phosphate dihydrate, disodium hydrogenphosphate and ethanol (99.5% pure) were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). BSA and RNase A were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). All chemicals used were of reagent grade and used as received.

Sample preparation

A stock protein solution containing 3.0 mg/ml protein in 5 mM citrate, 5 mM phosphate, pH 4.0 or 7.0 was prepared. A 130 μl aliquot of the stock protein solution was mixed with 1820 μl of a mixture comprising a buffer containing 5 mM citrate, 5 mM phosphate buffer and a 75% ethanol solution in the same buffer at respective pH. The ratio of the buffer and the 75% ethanol solution was varied to cover 0-70% ethanol concentration. The final protein concentration was 0.2 mg/ml. The protein solutions were incubated at room temperature in a turnover motion for 1 hours and centrifuged at 18,800 × g for 15 min. After centrifugation, the supernatant was used for the following measurements.

Absorbance measurement

The absorbance measurements were performed at room temperature using a V-630 UV-vis spectrophotometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan) and a 10 mm path-length quartz cell. The absorbance spectra of the samples in the supernatant were corrected by subtracting the spectra of the respective solvents.

Circular dichroism measurement

Far-UV circular dichroism (CD) measurements were performed at 25°C using a J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan) and a 1 mm path-length quartz cell. The CD spectra of the samples were corrected by subtracting the spectra of the respective solvents and converted to the mean residue ellipticity using the protein concentration of the supernatant.

Fluorescence measurement

Intrinsic fluorescence spectra of protein were determined at 25°C using a FP-6500 spectrofluorometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan). The emission spectra (3 nm slit-width) were recorded with excitation at 295 nm (3 nm slit-width) and corrected by subtracting the spectra of the respective solvents.

Native polyacrylamide gel electrophoresis (native-PAGE)

Native-PAGE was carried out according to the previous study [20] using an 8% Tris-Gly gel and a Tris-Gly tank buffer (25 mM Tris-HCl, 192 mM glycine, pH 8.3). The samples were mixed with an equal volume of sample buffer containing 50% sucrose, 0.01% (w/v) bromophenol blue and 125 mM Tris-HCl, pH 6.8. After gel electrophoresis, the gels were stained with silver nitrate. It should be emphasized that the native-PAGE analysis monitors irreversible changes that occur at pH 4.0 or 7.0 and in the presence of ethanol.

5.3. Results and discussion

BSA

I have examined here the effects of ethanol on the structure of BSA and RNase A at pH 7.0 and 4.0. First, the structure differences at these two pH values were examined for BSA. Figure 5.1A shows the UV absorbance spectra of BSA at pH 7.0 and 4.0, showing small differences between these two pH values. Such small differences could, however, be simply due to experimental errors, e.g., in protein concentration. Figure 5.1B shows the CD spectra of BSA at pH 7.0 and 4.0. While the spectral shapes were identical, the intensity appeared to be slightly weaker at pH 4.0. Nevertheless, it should be safe to state that protein structure is not significantly different between these pH values.

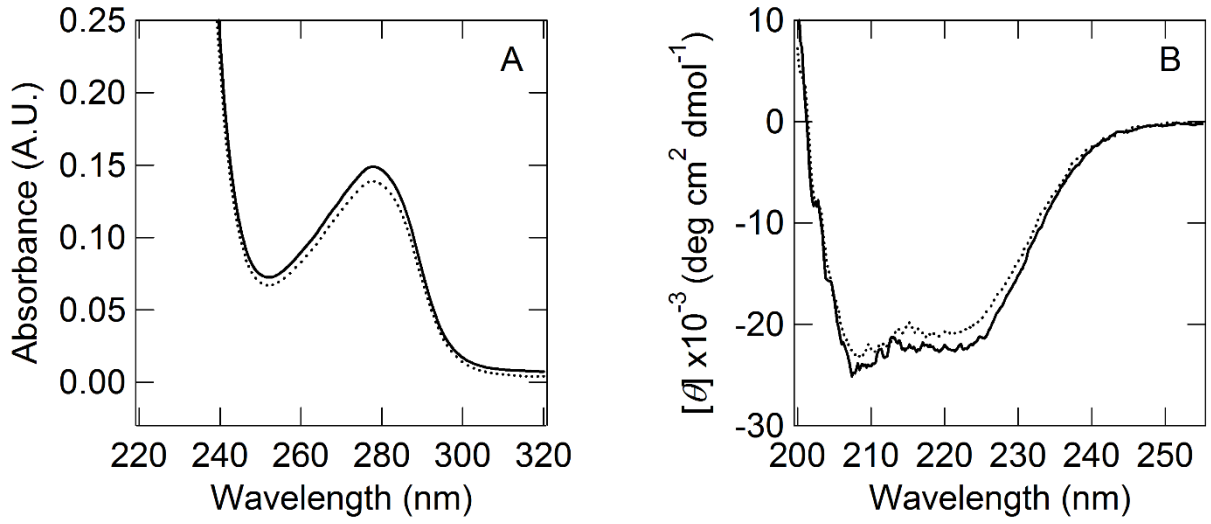

Figure 5.1. Absorbance (A) and far-UV CD spectra (B) of BSA at pH 7(solid line) and pH 4(dotted line)

Having established a similar structure at pH 7.0 and 4.0, the effects of ethanol on BSA structure were examined at pH 7.0. UV absorbance spectra at pH 7.0 are shown in figure 5.2A. There is little change in absorbance spectra between 0 and 40% ethanol, demonstrating no apparent changes in light scattering and hence associated state up to 40% ethanol. A large absorbance increase in the entire spectral region was observed at 50%, an indication of light scattering. Since the sample was spun before absorbance measurement, such light scattering indicates formation of soluble oligomers. The BSA sample used here contained several oligomer bands in addition to main monomeric band, as analyzed by native-PAGE shown in figure 5.2E. While no changes in native-PAGE pattern were observed at 50%, the intensity of each band significantly decreased. It thus appears that 50% ethanol increased the size of the soluble oligomers so that they no longer enter the 8% gel, leading to reduction of each of monomer and oligomer bands. Light scattering does not appear to be present at 60% ethanol, as seen by no apparent absorbance at 310-320 nm, implying that formation of soluble oligomers was replaced by precipitation: the absorbance was greatly reduced at 60% after centrifugation. This result was confirmed by native-PAGE showing only a small amount of monomer left: namely, all BSA forms were converted to precipitates. There seems to be no protein left at 70% ethanol based on absorbance and native-PAGE results.

Figure 5.2B shows the CD spectra at pH 7.0 as a function of ethanol concentration. Consistent with little changes in absorbance spectra up to 40%, the CD spectra were identical within experimental errors between 0 and 40% ethanol. The spectrum at 50% was also unchanged, different from the observed large increase in absorbance due to light scattering. This suggests that the soluble oligomers formed at 50% have intact secondary structure. A significant structure change appeared to occur at 60% ethanol, at which BSA showed considerable precipitation: note that accurate CD and absorbance measurements could not be done due to low protein concentration of the supernatant. Thus, while 60% ethanol resulted in reduction of protein solubility upon structure alteration, 50% ethanol enhanced inter-molecular interaction between the intact structures. There is no protein left at 70% for CD measurement. Tertiary structure of BSA at pH 7.0 was examined by fluorescence spectra.

Figure 5.2C shows the fluorescence spectra as a function of ethanol concentration. No significant differences were observed up to 50%, except for the effects of ethanol itself on the fluorescence properties of the tryptophans present in BSA. Such ethanol effects are clearly seen in figure 5.2D, in which both fluorescence intensity and wavelength maximum are plotted against ethanol concentration. The wavelength maximum gradually decreased with ethanol concentration due to reduced polarity of aqueous ethanol solution [21]. On the contrary, the fluorescence intensity sharply dropped at 50% and further at 60%. Although far UV CD spectrum at 50% showed little changes, the fluorescence intensity was significantly reduced. Such reduction may be related to the observed light scattering at 50%, perhaps due to fluorescent tryptophan involved in enhanced oligomer formation. The observed loss of fluorescence intensity at 60% is due to precipitation and hence loss of the protein. Since there is still protein left at 60% (see absorbance spectrum), however, nearly complete loss of fluorescence intensity suggests conformational changes. Thus, BSA at pH 7.0 undergoes no secondary structure changes up to 60% ethanol, but there may be changes in tertiary structure above 60% ethanol.

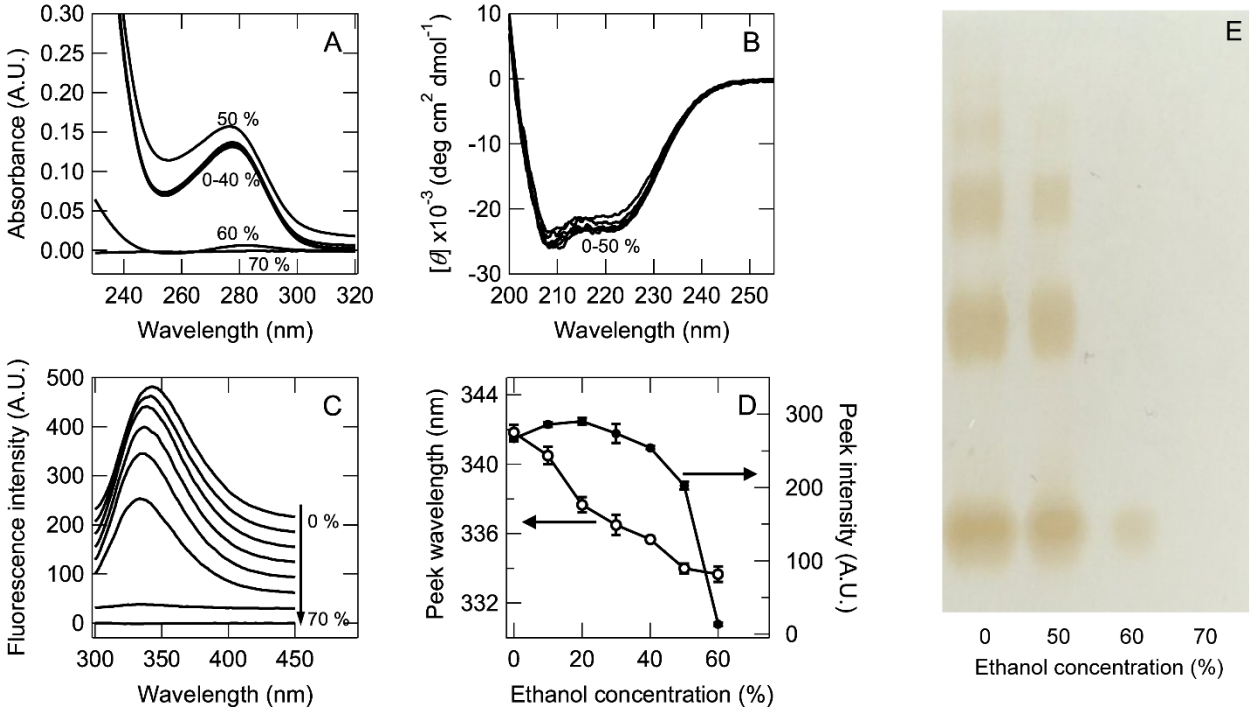

Figure 5.2. The effects of ethanol on the spectroscopic properties of BSA at pH 7. Absorbance (A), far-UV CD (B) and intrinsic emission fluorescence spectra (C) as a function of ethanol concentration. Each fluorescence spectrum was shifted upward by 30 A.U. for visual comparison. (D) Fluorescence peak maximum and peak intensity as a function of ethanol concentration. In (D), the measurements were performed three times, and the error bars depict the standard deviation of the mean. (E) Native-PAGE.

A contrasting picture emerges at pH 4.0, at which BSA is positively charged. Figure 5.3A shows the UV absorbance spectra of BSA at pH 4.0, in which no spectral changes were observed up to 60% (compare with the precipitation at 60% at pH 7.0, Figure 5.2A). Consistent with no changes in absorbance spectra up to 60%, native-PAGE pattern and band intensities appeared to be unchanged, as shown in figure 5.3E. A large light scattering was observed at 70%, at which extensive precipitation occurred at pH 7.0. It thus appears that BSA at pH 4.0 was more resistant to ethanol than at pH 7.0 against precipitation, but formed more soluble oligomers or aggregates: compare with the absorbance spectrum at 50% ethanol, pH 7.0. Native-PAGE (Figure 5.3E) showed decreased intensity of each band without appearance of new oligomers bands at 70%, suggesting that the oligomers formed are too large to enter the gel. Consistent with the UV absorbance data, CD spectra were unchanged up to 60%. CD spectrum at 70% was significantly different, similar to the result at 70% ethanol, pH 7.0. Such different secondary structure at 70% may be related to the observed extensive light scattering at pH 4.0, in contrast to the structure changes at pH 7.0 that resulted in precipitation. Similar to the

far UV CD spectra, the fluorescence properties were little affected by ethanol at pH 4.0. Only significant change was a sharp blue shift at 10-20% ethanol. It is not clear why 10 % ethanol caused a shift in wavelength maximum at pH 4.0 to the extent that required 60% ethanol at pH 7.0. In any case, it is evident that the response of BSA to the addition of ethanol is pH-dependent: namely, the charged state of BSA may be related to the way ethanol affects its structure and self-association.

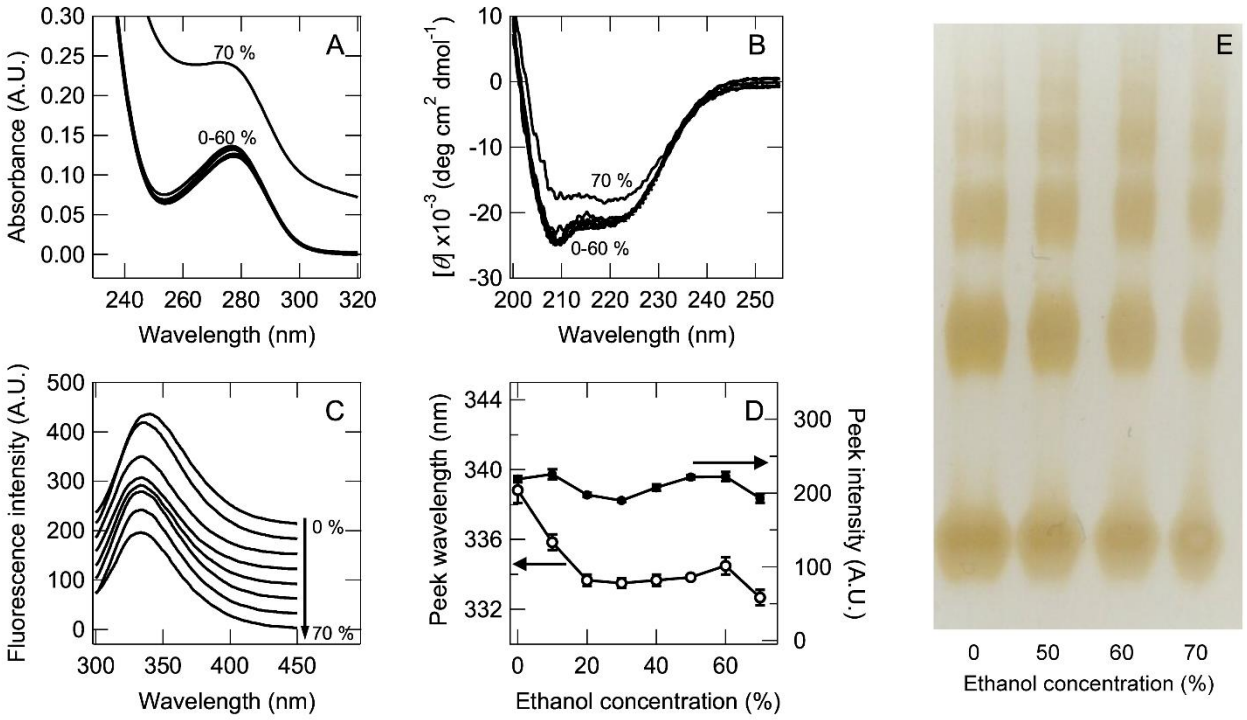

Figure 5.3. The effects of ethanol on the spectroscopic properties of BSA at pH 4. Absorbance (A), far-UV CD (B) and intrinsic emission fluorescence spectra (C) as a function of ethanol concentration. Each fluorescence spectrum was shifted upward by 30 A.U. for visual comparison. (D) Fluorescence peak maximum and peak intensity as a function of ethanol concentration. In (D), the measurements were performed three times, and the error bars depict the standard deviation of the mean. (E) Native-PAGE.

RNase A

A similar experiment was performed with RNase A, which, unlike BSA, is positively charged at both pH 4.0 and 7.0, with more net positive charges at pH 4.0. Figure 5.4A shows the absorbance spectra at pH 4.0 and 7.0, with no significant differences. Consistent with the absorbance spectra, the CD spectra were identical at pH 4.0 and 7.0 (Figure 5.4). Thus, there appear to be no significant structure differences at two pH values for RNase A as well.

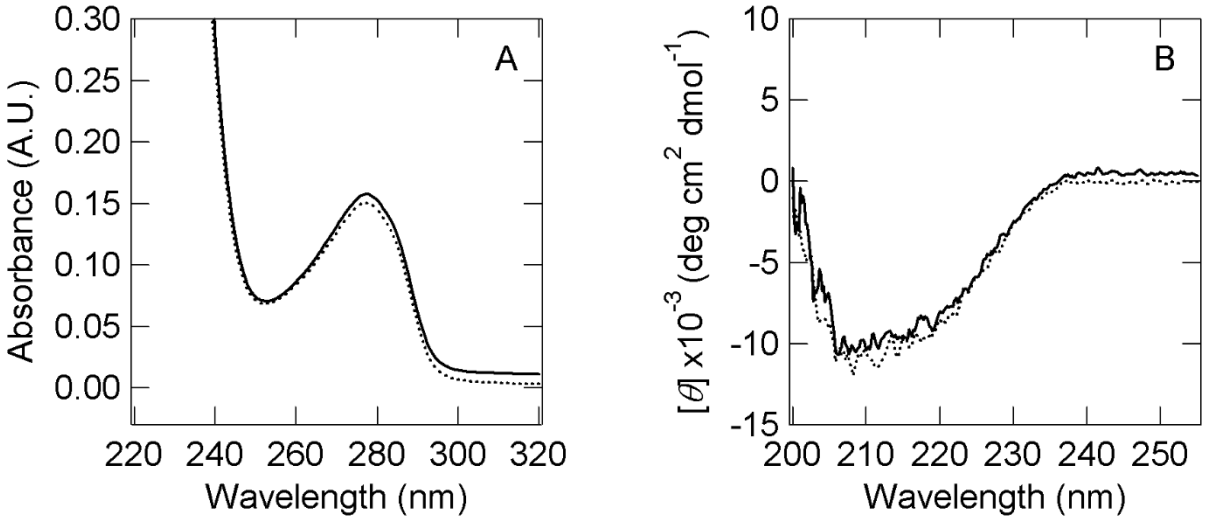

Figure 5.4. Absorbance (A) and far-UV CD (B) spectra of RNase A at pH 7 (solid line) and pH 4 (dotted line).

Even though RNase A structure appears to be independent of the charged state and is positively charged at both pH values, the response to ethanol was pH dependent. Figure 5.5 shows the results at pH 7.0, while figure 5.6 shows those at pH 4.0. At pH 7.0, the absorbance spectra were unchanged up to 60% and significant precipitation was observed at 70% ethanol as seen by the decreased absorbance of the supernatant. Interestingly, CD spectra were identical even at 70% (Figure 5.5B), suggesting that 70% ethanol simply enhanced protein-protein interactions in the native state, resulting in precipitation. At pH 4.0, the results of UV absorbance were essentially identical to those at pH 7.0 at 0-60% ethanol, as well as the CD spectra at 0-70%. No changes in secondary structure up to 70% and no absorbance changes up to 60% have occurred. However, a large increase in absorbance was observed at 70% due to light scattering. At pH 7.0, 70% ethanol resulted in precipitation of RNase A, while it resulted in formation of soluble aggregates at pH 4.0. The results with RNase

A clearly show salting-out effects of ethanol [22]. Namely, it can enhance protein-protein interaction without altering the structure. The observed differences in the type of protein-protein interaction between pH 4.0 and 7.0 may simply be the degree of self-association. RNase A is more positively charged and thereby has stronger charge repulsion at pH 4.0, which may prevent the protein from forming lager aggregates to precipitate.

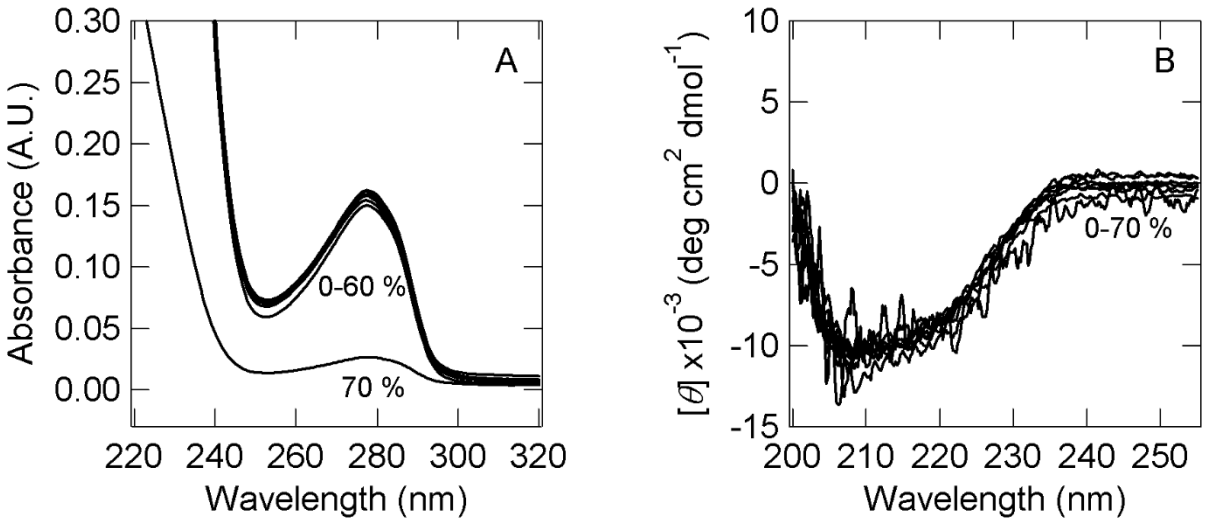

Figure 5.5. The effects of ethanol on the spectroscopic properties of RNase A at pH 7. Absorbance (A) and far-UV CD spectra (B) of RNase A as a function of ethanol concentration.

A question is then how ethanol enhances protein-protein interaction in the first place, while enhancing electrostatic repulsions. It has been shown that there is unfavorable interaction between charged solutes and low dielectric organic solvents, such as ethanol [22–25]. When such unfavorable interaction reaches a threshold value at certain organic solvent concentration, the charged solutes like protein phase-separate as a form of either soluble aggregates or precipitation [22,25]. There appears to be fine balance between inter-molecular charge repulsion and unfavorable solute-solvent interaction, which determines the extent of protein-protein interaction, i.e., soluble aggregates vs. precipitation.

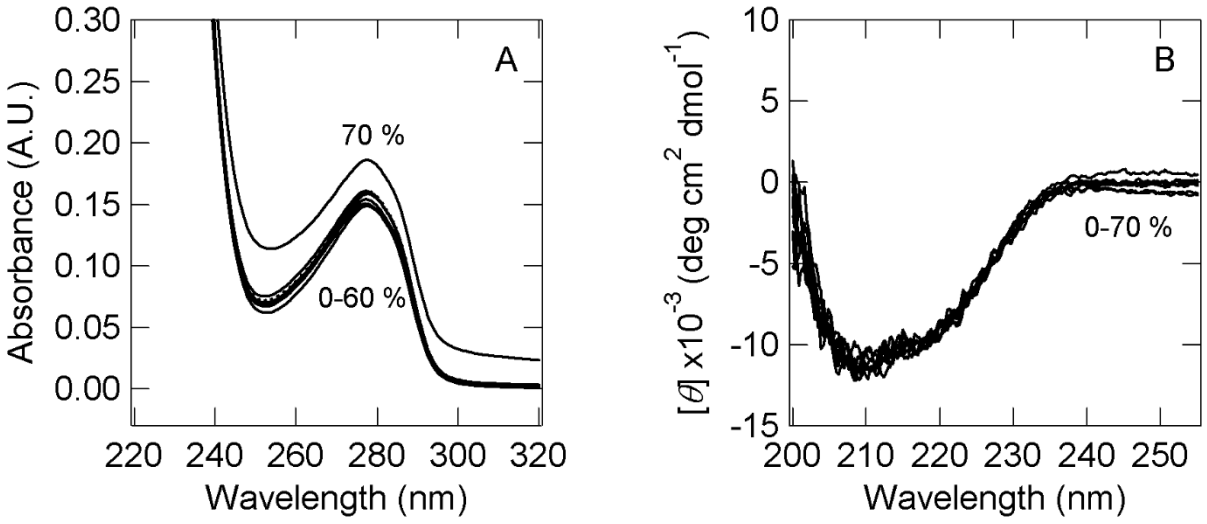

Figure 5.6. The effects of ethanol on the spectroscopic properties of RNase A at pH 4. Absorbance (A) and far-UV CD spectra (B) of RNase A as a function of ethanol concentration.

How do these results relate to inactivation of non-enveloped viruses? Non-enveloped viruses are composed of nucleic acids and proteins. This study showed that ethanol at high concentrations, above 50-60%, alters the structure or the association state of BSA and RNase A pH dependently and suggested a possibility that ethanol may inactivate non-enveloped viruses through its effect on proteins. Roberts and Lloid showed that 70%, but not 20%, ethanol inactivated poliovirus at pH 4.5 [5]. Requirement of such high ethanol concentration is consistent with the observation in this chapter. Namely, high ethanol concentration may irreversibly change the structure or the inter-molecular interaction of viral proteins, thereby leading to loss of infectivity. It would be of great interest to study the pH dependent effects of ethanol on additional model proteins and their impacts on viral proteins.

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Chapter 6.

General Discussion

Protein is a useful material owing to its functionality, and the pharmaceutical application of protein is a promising field. The agglomerating property of protein in solution is the main obstacle for its safe and economical use. In this thesis, I provide the knowledge of protein–cosolvent interaction as a method of stabilizing pharmaceutical protein. In this section, the overview, perspective, and future study of this thesis are described.

Arginine is widely used as an aggregation suppressor without denaturation of the protein. Although its application is versatile, it is mostly used with its counterion, chloride, to adjust the solution pH. To modulate the effect of arginine, six types of arginine salts were tested. As expected, the counterion changed the effects of arginine, such as the solubility of aromatic compounds and facilitation of refolding. Because solubility is an important factor in formulation, using different counterions may be useful technique to the low-molecular-weight drugs. Interestingly, the calculation of free energy suggested that the effects of arginine and counterion affect the solubility of propyl gallate independently. Thus, the effect of counterions on the solubility of simple compounds can be predicted. The counterion also modulated the effect of arginine on protein aggregation. However, the effect on protein is more complicated than that on simple compounds, indicating the presence of an additional factor affecting protein stability. Some research group suggested that ion-to-ion interaction can suppress protein aggregation using molecular dynamics simulation. Further study using solution chemistry analysis methods, such as X-ray diffraction, is needed to elucidate the influence of counterions on the effect of arginine.

In the pharmaceutical application of antibody, aggregation is a major concern because of its immunogenicity. Although knowledge of various cosolvents has been accumulated using some model proteins, there is no guarantee that the same effect will be obtained using the antibody because of its structural complexity. In this thesis, various cosolvents were tested to determine their effect on the aggregation of human immunoglobulin G (IgG) as a model antibody protein. Unlike a globular protein, the thermal aggregation pathway of IgG is divided into several stages: (i) the formation of soluble oligomers, triggered by the unfolding

process, which can be inhibited by a stabilizer, such as sugar, and (ii) the formation of insoluble aggregates through weak cluster–cluster interactions, which can be suppressed by arginine. The soluble oligomers could not be dissociated by the cosolvents used in this thesis, indicating the importance of suppressing the unfolding process in the stabilization of antibody. Thus, the investigation of a strong protein stabilizer is essential for the stabilization of antibody in the future. Moreover, it is known that other stress such as pH, agitation and freezing can induce the formation of soluble oligomer. Considering the cosolvent shows the different effect on each stress, further investigation for various type of stress is needed for the management of the pharmaceutical process. On the other hand, knowledge of the physical and chemical properties of the soluble oligomer is insufficient. For example, what driving force involves the formation of a soluble oligomer? Is a covalent bond, such as a disulfide bond, formed in the aggregates? Answering these questions will provide the critical information for establishing antibody stability.

The production cost of pharmaceutical proteins is higher than that of conventional low-molecular-weight drugs and needs to be reduced. The multidose formulation, which requires the addition of antimicrobial preservatives, such as benzyl alcohol, is one of the approaches to lower the cost. However, the antimicrobial preservative facilitates protein aggregation due to a hydrophobic interaction. In this thesis, trimethylamine N-oxide (TMAO) can suppress the benzyl-alcohol-induced aggregation of IgG. In the pharmaceutical industry, the cost reduction approach is needed from the up- to downstream process. Thus, these results will provide helpful information in the development of multidose formulations of proteins. It will be important to investigate the effect of TMAO on the destabilization effect of other antimicrobial agent such as *m*-cresol and phenol. Unfortunately, TMAO has not yet been approved by the US Food and Drug Administration and further study of the safety of TMAO is also needed.

In addition to the effects of TMAO, I found an interesting feature in the protein chemistry. Despite its higher structural stabilization effect, trehalose is less useful than TMAO for the prevention of benzyl-alcohol-induced aggregation. This discrepancy cannot be explained simply using the protein–cosolvent interaction and requires investigation of the cosolvent–cosolvent interaction. Although the interaction between cosolvents is usually overlooked by the simplification of the protein solution, it does not mean that cosolvents do not interact with each other; all components, such as protein, water, and cosolvents, interact with the various

combinations present. Therefore, formulation design that considers not only protein–cosolvent interaction but also cosolvent–cosolvent interaction may improve the stability of pharmaceutical proteins. Further study will be required using both experimental and computational methods to understand all the interactions that occur in solution.

Ethanol is conventionally used as a disinfectant that disrupts the membrane of enveloped viruses. However, a non-enveloped virus, covered with capsid protein rather than a lipid, is also inactivated by ethanol under specific conditions. To clarify the inactivation mechanism of a non-enveloped virus by ethanol, the effect of pH on the conformational changes and self-association of two model proteins were investigated. Although a structural discrepancy existed between capsid and model proteins, the results in this thesis suggest a hypothesis for the inactivation of a non-enveloped virus. Under conditions that cannot solubilize the protein, capsid would rather precipitate than alter its structure. Thus, the activity of a non-enveloped virus will be retained, and the non-enveloped virus will exert its toxicity after resolubilization. However, if the conditions allow the structure of capsid to be irreversibly altered, the protein can disperse. In short, the inactivation of the non-enveloped virus may require a specific time to interact with ethanol molecules in solution. Fortunately, current biotechnological progress has made it possible to generate an artificial capsid. A future study using the capsid protein itself may help to understand the disinfection mechanism of the non-enveloped virus.

Chapter 7.

Concluding Remarks

This thesis investigated the application of protein–cosolvent interaction on pharmaceutical applications. Chapter 2 showed that the effect of arginine could be modulated by altering its counterion. Considering the versatile function of arginine, the adjustment of the counterion will be a useful method for various applications, including pharmaceutics. Chapter 3 described the effect of the cosolvent on the thermal aggregation of IgG. The presence of a soluble oligomer state will present a new approach for a stabilization strategy for antibodies using a cosolvent. Chapter 4 presented a method for aggregation suppression induced by an antimicrobial preservative. Results in this chapter also indicated the importance of the interaction of cosolvents for protein stability. This viewpoint will provide suggestive insights for the pharmaceutical application of cosolvents. Chapter 5 showed the pH-dependent behavior of BSA and RNaseA in an ethanol solution, suggesting that the denaturation process is important for the disinfection mechanism of non-enveloped viruses.

The stabilization of pharmaceutical protein solutions is a complex problem because of its diversity. Each pharmaceutical product should be produced based on various considerations, including target disease, dose, molecular mechanism, country regulations, and administration method. An improvement in the process from industry production to the administration of the final protein solution in the hospital is necessary to deal with protein instability in solution. Various promising technologies from diverse research fields exist. In material science, surface modification can reduce the adsorption of protein. In mechanical engineering, methods to reduce vibration can reduce protein aggregation induced by shaking during transport. In polymer science, the protein–polyelectrolyte complex can protect the protein from some types of stress. Selecting a combination of the technologies illustrated above will be a momentous decision for the pharmaceutical industry in the future. Along with such technical progress, cosolvents will continue to be a fundamental element for pharmaceutical solution formulation due to their simplicity and compatibility. The findings in this thesis represent a good base knowledge for the pharmaceutical industry with regard to improving protein stability in solution.

Although much research has been conducted, the application of cosolvents still has room for improvement. A vast library of compounds used for drug discovery already exists, and the development of a similar cosolvent library is required. The development of a screening method for protein stability will facilitate the discovery of a new type of protein stabilizer. Computational methods for understanding and predicting the effect of cosolvents on a protein solution would also be useful. Considering the timescale of protein dynamics, current experimental technology cannot reveal the complete behavior of a protein in solution. To overcome this facility and theoretical problem will enhance the importance of protein science. These cross-sectional approaches will be necessary for future pharmaceutical research.

List of Publications

Related Publications

(1) ○**Shunsuke Yoshizawa**, Tsutomu Arakawa, Kentaro Shiraki. Dependence of ethanol effects on protein charges, *International Journal of Biological Macromolecules*, 68, 169-172, 2014.

(2) ○**Shunsuke Yoshizawa**, Tsutomu Arakawa, Kentaro Shiraki. Effect of counter ions of arginine as an additive for the solubilization of protein and aromatic compounds. *International Journal of Biological Macromolecules*, 91, 471-476, 2016.

(3) ○**Shunsuke Yoshizawa**, Tsutomu Arakawa, Kentaro Shiraki. Thermal aggregation of human immunoglobulin G in arginine solutions: contrasting effects of stabilizers and destabilizers. *International Journal of Biological Macromolecules*, 104, 650-655, 2017.

(4) ○**Shunsuke Yoshizawa**[1], Shogo Oki[1] Tsutomu Arakawa, Kentaro Shiraki. Trimethylamine N-oxide (TMAO) is a counteracting solute of benzyl alcohol for multi-dose formulation of immunoglobulin, *International Journal of Biological Macromolecules*, 107, 984-989, 2018.

[1]These authors contributed equally to this paper.

Other Publications

(1) Eisuke Takai[1], ○**Shunsuke Yoshizawa**[1], Tsutomu Arakawa, Kentaro Shiraki. Synergistic solubilization of porcine myosin in physiological salt solution by arginine, *International Journal of Biological Macromolecules*, 62, 647-651, 2013.

[1]These authors contributed equally to this paper.

(2) Eisuke Takai, Tsuyoshi Kitamura, Junpei Kuwabara, Satoshi Ikawa, ○**Shunsuke Yoshizawa**, Kentaro Shiraki, Hideya Kawasaki, Ryuichi Arakawa, Katsuhisa Kitano. Chemical modification of amino acid by atmospheric-pressure cold plasma in aqueous solution. *Journal of Physics D:Applied Physics*, 47, 285403, 2014.

(3) Takumi Miyatake, ○**Shunsuke Yoshizawa**, Tsutomu Arakawa, Kentaro Shiraki. Charge state of arginine as an additive on heat-induced protein aggregation. *International Journal of Biological Macromolecules*, 87, 563-569, 2016

(4) Tomohito Nakayama ○**Shunsuke Yoshizawa,** Atsushi Hirano, Kentaro Shiraki, Muneaki Hase. Vibrational Energy Transfer from Photo-Excited Carbon Nanotubes to Proteins Observed by Coherent Phonon Spectroscopy. *Appl. Phys. Express*,10, 125101, 2017.

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